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Molecular Structure of Bacterial Plasmids

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INTRODUCTION AND DEFINITIONS

During the past few years, an increasing number of bacterial properties have been described which behave in such a way as to suggest that their genetic control is not chromosomal. There has been a natural tendency to attribute to many of these properties the same genetic and molecular basis as has been derived from considerably more extensive studies of such related elements as the F sex factor (136) and the temperate bacteriophage λ

(113). Since the application of the terminology developed for F and λ to these other genetic elements often leads to specific implications, definitions of terms are particularly important.

Thus, an *extrachromosomal element* is here defined as a genetic element which is physically separate from the chromosome of the cell and is able to be perpetuated stably in this condition. The term therefore embraces such elements as the P1 prophage but would exclude wild-type λ prophage or the deoxyribonucleic acid (DNA) of abortive transductants. As presently used in bacteria, the term "extrachromosomal element" is synonymous with the term "*plasmid*," which is further defined as an element which is nonessential for the growth of normal cells of the host species, so that under most conditions it may be gained or lost without lethal effect. Those plasmids which have been shown to be able to occupy a chromosomal site are referred to as *episomes*. Thus, whereas a plasmid may or may not be an episome, all episomes are plasmids. Since the definition of "episome" implies a genetic relationship between the plasmid and the host cell, the same element may be an episome in one host but a plasmid in another. It has often been implied that plasmids are those episomes where a chromosomal location in one or another host has not so far been demonstrated. It should be borne in mind, however, that there would appear to be no a priori reason that a plasmid must be able to integrate into, or have genetic homology with, the chromosome of any host cell.

Plasmids may be conveniently classified into two major types, infectious and noninfectious plasmids. *Infectious* (or *self-transmissible*) *plasmids* (e.g., F, ColIb) control the establishment in the host cell of a "donor state" (through such physical features as *sex pili*), to provide a mechanism of conjugation that permits their transfer from the host cell into another cell, the recipient, which is thereby itself converted into a donor. Those infectious plasmids that can also promote the transfer of other genetic material, either chromosomal or plasmid, are known as *sex factors*. The question at the moment remains open whether or not all infectious plasmids have this property. *Noninfectious plasmids* (e.g., ColE1) are not able to set up the donor state and require either that a sex-factor plasmid be present in the same cell for their conjugal transfer or that transfer be effected by a transducing phage. The plasmid nature of a noninfectious plasmid is thus more difficult to establish and is often inferred by determining that its transmission is unlinked to any chromosomal marker. However, conjugal transfer of both chromosomal and nonchromosomal material to the same recipient cell can occur during the course of normal mating conditions (*see* 42). This may be mistaken for linkage of a plasmid with chromosomal markers (4) and can be distinguished by observing conjugal transfer from a number of Hfr donors with differing "origines" of transfer, in which case true linkage is unaffected whereas an extrachromosomal element will show a constant time of entry, by all Hfr strains (42). Both types of plasmid can be transmitted efficiently in a generalized transducing phage system (10, 12, 83, 105, 237).

It will not be the intention of this review to consider in any detail many of the genetic and physiological properties of bacterial plasmids which have been extensively dealt with in a number of recent reviews and symposia (71, 168, 178, 247, 252), nor will the physical aspects of those plasmids with no clear genetic or physiological function (30, 54, 125, 154, 206) be examined, nor yet those that can be regarded as phages (166, 214). In general, the main emphasis will be on the physical characteristics of those plasmids, with clearly defined properties, that are not phages and, wherever possible, the relationship of their genetic and molecular properties. Other reviews focussing on the physical aspects of some of these plasmids have recently appeared (63, 88, 111).

It now seems possible that many plasmid genes do not have chromosomal counterparts. Thus, there is clearly a need for distinguishing between plasmid and chromosomal genes which, though phenotypically similar, may be functionally quite distinct. In fact, the view expressed by Demerec et al. (66) that "loci on plasmids and episomes are not different in kind from loci on the chromosome" could be disputed. Thus, the symbols used in the original publications for plasmid determinants will be continued, in spite of the fact that frequently these do not conform to the standard triletter lower case italics (66).

SOME PROPERTIES OF BACTERIAL PLASMIDS

Most of the bacterial plasmids extensively studied have been those originating in strains of *Enterobacteriaceae*, although recently an increasing number have been characterized from staphylococcal strains. In enteric bacteria, the identification of the classical plasmid F has been followed in recent years by the dis-

covery of two other major groups of plasmids: the colicinogenic (Col) plasmids and the infectious drug-resistance (R) plasmids. Most of these plasmids also give rise to the donor state in their host cells with the result that the F sex factor was for some time taken as a model for all plasmids. However, during the last few years it has become increasingly clear that these other groups of plasmids, although showing many of the important characteristics of the F factor, differ in other major respects. Some of these differences are outlined below.

Chromosomal Integration

A major feature of F is its ability to integrate within the chromosome of the host strain *Escherichia coli* K-12, by which property it was characterized as an episome and, together with λ, formed the basis for the original definition of that element by Jacob and Wollman (135). (It should be noted that wild-type λ undergoes lethal [vegetative] replication and is thus unsuitable for classification as a true bacterial plasmid.) However, integration may in fact be a non-essential property of plasmids, since a number of bacterial plasmids, for example, the colicinogenic factors ColIb or ColE1, do not appear to integrate within the chromosome of at least one of their common hosts, *E. coli* K-12. Transmission of ColIb or ColE1 from a series of Hfr K-12 donors is thus independent of any chromosomal marker (42, 171). Nevertheless, ColIb is an effective sex factor in K-12 (41), and its ability to transfer chromosomal markers has sometimes been used to imply chromosomal integration. If this were true, and integration via normal recombination were blocked by the presence of a *recA* (33) mutation in a donor strain, then we would expect that chromosomal transfer would be similarly blocked. We have shown that, although in the case of those transfer factors such as F, which are known to integrate, the use of *recA* donors considerably reduces chromosomal transfer, nevertheless, a small level of transfer (about 1 recombinant per 10^8 donor cells) remains (45). This level does not appear to be due to leakiness of the *recA* mutation, since there is a loss of polarity of marker transfer by F′ factors from *recA* donors compared to transfer from *recA*$^+$ donors (whereas, residual activity, if mediated via integration by recombination, would be expected to maintain the polarity of normal transfer). More importantly, chromosomal transfer by ColIb is independent of *recA* function and may therefore be concluded to be independent of the integration of ColIb into the chromosome (45), although the possibility of integration via a mechanism related to the λ integrase system (99), or some other mechanism (82), is not eliminated. More recent evidence, using *E. coli dnaA* mutants which are temperature-sensitive (ts) in the *initiation* of their chromosomal DNA replication (117, 118), supports the idea that ColIb does not integrate in *E. coli*. Revertants of such ts mutants (but not of the "fast-stop" chain elongation DNA replication mutants), able to grow at the normally nonpermissive temperature, are found at an increased frequency from ts strains carrying an F sex factor. These revertants appear to arise as a result of a mechanism (termed "integrative suppression") resulting from integration of the F sex factor in the chromosome, so that a new initiation site for chromosomal replication is provided within the integrated sex factor (175). Such revertants cannot be isolated from the same ts mutants harboring a ColIb factor (173), or indeed one of several R factors of the fi$^-$ type (173; T. Arai, *personal communication*), implying that these plasmids are not able to integrate in *E. coli* K-12.

Since the time that an extrachromosomal nature was suggested for such potentially lethal elements as the ColIb plasmid (43, 171), it has also been established that certain temperate phages, such as P1, set up their prophage state without integration of the prophage genes within the chromosome (126). It may well be, therefore, that episomes are a special class of plasmids having an additional property of being able to integrate within certain host chromosomes, and it appears an open question at the moment whether chromosomal integration has any important bearing on either the evolution or the stability of the plasmid-host relationship.

Fertility Repression

It has been shown that many sex factors do not generally express the properties of the donor state in all cells of a host strain (168, 171, 223), F again being an exception to the rule. The fertility properties of most sex factors are generally repressed in the majority of cells, and their fertility has been concluded to be regulated by an operator-repressor type system (168, 172). (Although a more recent analysis of a number of transfer-defective mutants has led to a more complex model, in essence it may be similar [1, 2, 80, 132, 246, 248, 249].) Mutations to derepression of fertility have been identified and have been shown

to behave genetically as of two major types, consistent with the idea that one type (which is recessive) occurs in a regulator gene producing a diffusible repressor-like substance, and the other type are due to mutations at the site of action of this repressor (the operator) and are thus dominant (119, 167). F appears to be defective in producing a repressor, since it is sensitive to the putative repressors of some repressed factors in biplasmid hosts (168, 239). [However, among a series of mutants of an F'*lac* factor selected as insensitive to this repression, two subgroups are found, one dominant and another recessive, when present in a triplasmid cell containing the repressing plasmid, parental F'*lac* and the F'*lac* mutant. From this it has been argued that repression is complex and requires at least two molecules, one (which is plasmid-specific) produced by all plasmids including F, and another, produced by self-repressed factors, which can also act on F (80, 246).]

The class of R factors able to repress F have been termed fi^+ (fi = fertility inhibition) and those R factors which do not repress the fertility of F are termed fi^- (239). Similar groups exist within the colicin factors. Some, such as the ColB factors, repress F (92, 109, 190), whereas others, such as ColIb, do not have this property (171).

As one manifestation of the donor state, specific hairlike processes termed sex pili or sex fimbriae are elaborated on the surface of the host cell (23). Some workers have concluded that the genetic material is transferred from the donor to the recipient cell via the pilus (23), but even though pili have been shown essential for the process of conjugation (*see* 56), this conclusion is an inference which rests at best on indirect evidence (186). Infectious plasmids appear to determine one of at least two distinct classes of pili. The factors termed F-like synthesize F pili which are similar in their sites for adsorption of those same male-specific phages (now more properly termed F-specific phages, e.g., MS2, R17, f2, $Q\beta$, $\mu 2$, fd) which adsorb to pili synthesized by the F factor. In contrast, I-like factors form I pili which do not carry sites for adsorption of the F-specific phages, but carry sites for adsorption of another group of phages termed *I-specific* (since they were first described for the ColIb factor) which do not in turn adsorb to F pili (168).

Until recently, the subdivision of F-like factors appeared to be identical with that of fi^+ factors, and I-like factors appeared to be of the fi^- factor type. However, it is now clear that many fi^- factors are not I-like (20, 58, 141, 233), some being reported to produce yet a third type of pilus (141). (It has also been claimed that, although most fi^- factors isolated in England are I-like, the majority of fi^- factors arising in Japan are not I-like [58].) Moreover, certain I-like plasmids have been reported to repress the fertility of the F factor when present in the same cell (they are fi^+; *see* 101), whereas some self-repressed F-like plasmids (e.g., ColB3; Clowes et al., J. Gen. Microbiol. **55**, proc. iv, 1965) do not repress F-pili formation (they are fi^-). These variations probably indicate that the interpretation of fertility regulation is more complex than a simple repressor-operator interaction (80, 109, 249).

Incompatibility

Usually two isogenic plasmids cannot be stably maintained in the same bacterial cell. Thus, an F'*lac* plasmid cannot be stably transferred to an Hfr strain (69), and, if transferred to an $(F'gal)^+$ strain, either the F'*lac* or the F'*gal* is perpetuated in individual cells, but not both (65). This phenomenon, termed *plasmid incompatibility*, is distinguished from *entry exclusion*, a change on the surfaces of plasmid-containing cells which inhibits the transfer of a related plasmid (76, 178). Incompatibility can also occur between pairs of plasmids which are not isogenic, e.g., ColV2-K94 and F (138, 160); F and ColV3-K30 (160); ColB2-K77 and 222/R (92, 109), and is usually a polarized process, one plasmid (the first of the pairs shown above) excluding the other, irrespective of which is the resident and which the superinfecting plasmid (109, 160). It has been reported that pairs of fi^- or of fi^+ R factors are incompatible, whereas an fi^- and an fi^+ plasmid can stably coexist in the same cell (194, 239). However, more recent reports show that, although all fi^+ R factors appear to be incompatible, several compatible groups have been found among fi^- R factors (20, 58). Incompatibility is usually interpreted as indicating close interrelationship, and it may indicate some common evolutionary origin of those factors involved. Clearly, until the process of plasmid replication and its regulation is more clearly understood, the basis for incompatibility is likely to be obscure.

Elimination

Plasmids may be lost spontaneously from host strains, and this loss may be increased by certain treatments (termed "curing"), in-

cluding the use of intercalating dyes (acridines [46, 116, 236] or ethidium bromide [21]), rifampin (16, 35, 137), thymine starvation (46, 160, 188a), crystal violet (203) or surface-active agents (131, 208, 222). Different plasmids vary considerably in their ability to be "cured," and this does not seem to be dependent upon such properties as sex-factor activity. For example, both ColE1 and ColIb are completely refractory to curing by acridine orange, which is most effective on F (46). Curing of F by acridine orange (AO) is restricted to the autonomous state, integrated F being refractory (116). Curing of autonomous F by AO has been shown to be due to the inhibition of F replication (120).

Replication and Segregation (Membrane Site Model)

Many current ideas on plasmid replication and segregation originate in a model first proposed by Jacob, Brenner, and Cuzin in 1963 (133). Although it now seems likely that some aspects of this model may be oversimplified, it still remains the basis of most ideas interpreting replication, segregation, and other important properties of plasmids. The hypothesis was proposed that those DNA molecules that are capable of replication (termed "replicons") are circular in structure and carry at least two gene loci controlling their replication; at one locus on the replicon is located a regulator gene which produces a diffusible substance (*initiator*) acting upon the second locus, an operator of replication (*replicator*), to permit DNA replication to be initiated from that point; regulation of replication is thus postulated to be under *positive control*. Fragments of DNA lacking these genes were presumed incapable of replication and were suggested to be characterized by such fragments of bacterial DNA as exist in abortive transductants. In the case of such elements as λ, in which vegetative replication is unregulated and finally leads to the lysis of the cell, the regulated (prophage) state is set up by integration of the λ genome within the chromosome of the host cell. By this means, replication of the λ prophage genome is coordinated with the replication of the host chromosome. Replication of the F plasmid, when in the autonomous (F^+) state, also appears to be regulated so that there is only one copy of the plasmid per chromosome, although in this state F is not integrated within the host chromosome but is, nevertheless, quite stable. To explain its regulated replication and stable inheritance, it was proposed that the F replicon was attached to a cellular site to which the chromosomal replicon was also attached. This attachment site, suggested to be located on the cell membrane, would control and transmit signals leading to initiation of replication of both chromosome and all attached plasmids. During each cell generation, the membrane site with the attached plasmids would duplicate so that, at cell division, a copy of the membrane site with attached chromosome(s) and plasmid(s) would be transmitted to each daughter cell, thus ensuring the stable inheritance of all cellular characters whether plasmid or chromosomally controlled. Incompatibility between isogenic plasmids was explained as due to a limitation to a single plasmid membrane site leading to competition for this site, so that only one of two isogenic plasmids could attach and be stably inherited. Incompatibility between apparently unrelated plasmids was suggested to be due to the use of the same replication site leading to similar competition, whereas unrelated and compatible plasmids were presumed to have distinct membrane-attachment sites. This model therefore offered an attractive yet simple explanation of the way that plasmids could be stably inherited even though they were present in as few copies as one per chromosome, in addition to suggesting a basis for the phenomenon of incompatibility.

Several experiments support the idea of a "segregation unit" of chromosome and F factor. Cuzin and Jacob (57) investigated the effect of a period of seven generations of growth at an elevated temperature on an F'*lac*$^+$ *E. coli* strain during which chromosomal replication occurred, but F factor replication was blocked by using a ts mutant F factor unable to grow at this temperature. The nonreplicated F factor was found to be segregated into only those cells to which chromosomal DNA which had been synthesized before F factor replication was inhibited was also segregated. More recent experiments have shown a similar physical association during growth and cell division between an F' factor and chromosomal DNA following inhibition of F' replication by AO (120).

However, several aspects of plasmid replication and incompatibility are difficult to explain on the membrane-replicon model. One aspect that has been emphasized (189) is that incompatibility of cells to a superinfecting F' element leads to the interpretation that F is

attached to its membrane-attachment site in Hfr cells as it is in F^+ or F′ cells, yet in a number of such Hfr strains, replication of the chromosome-F continuum is not controlled by F (31, 251). Another anomaly is that, although ColV2 is incompatible with F and excludes autonomous F, it can stabilize in an Hfr strain where its replication and colicinogeny properties appear to be normal, and the transfer properties of colicin factor and chromosome are unchanged (160). These and other objections could be overcome if it were assumed that regulation is effected through a repressor, in an analogous way to which superinfecting λ replication is controlled in λ lysogens. Such a system of *negative control* may be superimposed on the positive control through an initiator. For example, Pritchard et al. (189) have proposed that initiator is made constitutively by the host cell, but an inhibitor of this initiator is synthesized by replicon genes in a discontinuous way, a burst being transcribed soon after initiation of each replication cycle, inhibiting initiation of a further cycle of replication until the concentration of the inhibitor falls by dilution due to cell volume increase during growth.

GENETICS AND LINKAGE OF PLASMID-BORNE GENES

In those cases where a number of genetic properties of the same bacterial cell appear to be extrachromosomal, as shown by their ability to be transferred independently of chromosomal markers both by conjugation and transduction and by their ability to be gained or lost from the cell either spontaneously or after "curing," it becomes important to know whether these properties are carried on a single plasmid structure or whether more than one element is involved (106, 107, 170, 241). The genetic data of a number of plasmid-borne genes are shown in Table 1, separated into two clear-cut groups. To avoid the structural implications arising from previously used terms such as "associated" and "dissociated," which have been used in some instances (52, 111, 200) to refer to what will later be inferred to be cointegration and recombination release of two plasmid-gene groups, and in another instance (9) to designate what may well be a completely different mechanism that leads to conjugal cotransfer of two plasmid-gene groups, I have denoted these plasmid-gene groups as either "cointegrates" or "aggregates."

TABLE 1. *Transfer and loss of plasmid genes*[a]

Plasmid	Conjugal cotransfer		Coelimination or loss		Cotransduction[b]	
PLASMID CO-INTEGRATES						
F′-Lac	100%(60/60)F^+Lac^+ 0%(0/60)F^+Lac^- 0%(0/60)F^-Lac^-	(45)	100%(40/40)F^-Lac^- 0%(0/40)F^-Lac^+ 0%(0/40)F^-Lac^-	(46)	?	
ColV2 or ColV3 (Col^+TF^-)	100%(151/151)Col^+TF^+ 0%(0/151)Col^-TF^- 0%(0/151)Col^+TF^-	(45)	100%(20/20)Col^-TF^-	(160)	?	
F·ColB·ColV·*trp*·*cys*	>90% (F·ColB·ColV·Trp·Cys)$^+$	(84)	>90% (F·ColB·ColV·Trp·Cys)$^-$	(84)	c.90% (F·ColB·ColV·Trp·Cys)$^+$	(84)
222/R4 (Su,Sm,Cm,Tc)[r]	100%(RTF$^-$,Su^r,Sm^r,Cm^r,Tc^r)	(235)	100%(RTF$^-$$Su^s$,$Sm^s$,$Cm^s$,$Tc^s$)	(236)	100%(RTF$^+$,Su^r,Sm^r,Cm^r,Tc^r)	(237)
Δ-T	100% Δ^-T^R	(10)	?		100%(22/22)Δ^-T^R	(10)
PII (pen-mer-ero)	N.A.		c.90% pen^r, mer^r, ero^r	(181)	c.99% (4875/4880)pen^r; ero^r	(181)
PLASMID AGGREGATES						
F,ColE1	100%F^+E1^+ (2%F^+E1^-;2%F^-E1^+)	(43)	<0.01%F^-E1^- (0/290) 100%F^-E1^-(290/290)	(46)	?	
F,ColE2	1%F^+E2^+ 99%F^-E2^- (0.1%F^-E2^+)	(43)	?		?	
Δ,S	1%Δ^-S^R 99%Δ^-S^S (0.1%Δ^-S_R)	(9)	?		0%(29/29)Δ^-S^R	(10)
Δ,A	4%Δ^-A^R 96%Δ^-A^S (0.4%Δ^-A^R)	(9)	?		0%(16/16)Δ^-A^R	(10)

[a] TF represents transfer factor activity of colicin factor; RTF (or Δ) represents transfer activity of R factor; parentheses in conjugal cotransfer of plasmid aggregates are results of interrupted matings.

[b] All transductions using P1.

In the group of plasmid genes termed cointegrates, conjugal cotransfer and coelimination (either spontaneous or by curing) is usually seen, although partial transfer or partial loss is occasionally observed. (In this group, cotransduction is also the rule rather than the exception. It is only expected between pairs of genes situated near enough on the same DNA molecule to be incorporated in the same bacteriophage particle, but when it does occur, it has been taken as the most reliable genetic criterion for the location of two plasmid genes on the same molecular structure. However, in those cases where the DNA content of a plasmid is considerably less than the DNA of the transducing phage genome, it has been suggested that the DNA of another physically distinct plasmid may be incorporated within the same transducing phage particle [102]. This conclusion, if validated, would indicate that cotransduction of plasmids cannot be considered as absolute evidence of linkage.)

In contrast, the genes shown in the group of "plasmid aggregates" are typically transferred independently by conjugation, are also lost independently, and cotransduction has not been observed. Within this group are included examples of what would normally be regarded as independent pairs of plasmids, such as F and ColE1, which, although found independently in nature, can be established in the laboratory in the same host cell. In addition, less clearly defined and naturally occurring systems such as ΔS and ΔA of Anderson and Lewis (9; *see* p. 382) are included. In the plasmid aggregate group, conjugal cotransfer can occur, even with high frequency. For example, after a normal mating procedure with F^+ColE1^+ donors, almost 100% of the recipient cells acquire both F and ColE1 (43). Only if resort is made to interrupting the mating after short periods of contact (data shown in parentheses in Table 1) can cells be found to which either one or other factor has been independently transferred (43). Nevertheless, treatment of donor cells with AO gives rise to an almost complete elimination of the F factor without any observable curing of the ColE1 factor (46), indicating physical independence of the two plasmids. Thus, during F transfer, the probability of independent ColE1 transfer during the same period of mating is very high. Conjugal cotransfer can therefore also be seen to be an unreliable criterion of genetic cointegration, although this implication has frequently been drawn (106, 169, 170, 241) or, a less clearly defined structural association has been inferred (5, 7, 9). However, just as chromosomal mobilization by ColIb may occur without ColIb integration in the chromosome (45), so transfer of ColE1 by ColIb (43, 223) or by F may be brought about by a mechanism which may not require direct physical interaction between the two genetic elements.

The behavior of the plasmids F and ColE2 are more typical of plasmid aggregates. ColE2 is not transferred to recipient cells at anything like the high frequency of ColE1, so that after the normal 2-hr period of conjugation, although about 99% of the recipients acquire the F sex factor, only about 1% in addition acquire ColE2, and it is again necessary to resort to interrupted mating in order to isolate the small number of cells (0.1%) that acquire ColE2 alone (43). Some of the R-factor systems investigated by Anderson and co-workers, for example those involving the transfer factor Δ and the drug-resistance markers S or A (9), show a close parallel with F^+ColE2^+ donors in conjugal transfer. From Δ^+S^+ donors, most of the recipients receive only the transfer factor Δ, a small number acquire in addition the drug-resistance marker S, and only with interrupted mating is transfer of S in the absence of Δ observed. ΔA behaves similarly to ΔS with a slightly more efficient cotransfer of Δ and A (9).

Circular genetic maps have been proposed to account for the data of some members of the plasmid group referred to as "plasmid cointegrates." Watanabe (230) suggested a circular linkage map for the R factor 222 (Fig. 1) as one structure to satisfy the results of transduction studies with phage P22. Similar maps have been proposed for the composite F-ColV-ColB-*trp-cys* plasmid by Fredericq (84) and for the PI penicillinase plasmid in *Staphylococcus aureus* by Novick (179; Fig. 2), both from the results of deletion analyses. In each case, the genetic data are consistent with the integration of a group of genes within a single molecular structure. However, there has been some controversy as to whether the genetic nature of the plasmid aggregates, particularly some of the Δ-mediated drug resistance factors investigated by Anderson and co-workers (7, 233, 238), can be similarly represented.

MOLECULAR MODELS FOR PLASMIDS AND THEIR IMPLICATIONS FOR THE EVOLUTION OF R FACTORS

It has often been implicitly and explicitly assumed that the origin and evolution of R factors is equivalent in many ways to that of F' factors (63, 231, 233). A transfer factor was proposed to integrate in the bacterial chromosome (as in the formation of an Hfr strain [209]) near to a chromosomal gene mutated to

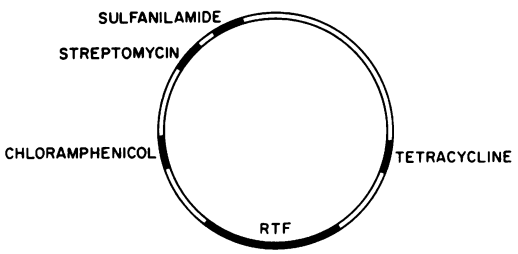

FIG. 1. *Circular genetic map of the 222/R factor proposed by Watanabe (230) on the basis of transduction data.*

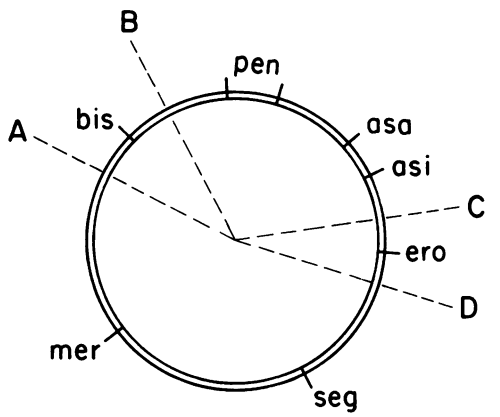

FIG. 2. *Proposed structure of the PI penicillinase plasmid from Staphylococcus aureus (179). The genes pen, ero, bis, asa, asi, and mer control resistance to penicillin, erythromycin, bismuth, arsenate, arsenite, and mercury, respectively, and the seg gene controls segregation. Mutants were mapped as shown by genetic deletion analysis. The contour length of the intact plasmid measured 9.4 μm, and the deletion mutants having lost the BCD and ABC regions have contour lengths of 8.1 μm and 7.3 μm, respectively.*

drug resistance. On release of the factor from the chromosome, this adjacent bacterial region would be incorporated as part of a newly created R factor. In this way, a factor could build up resistance consecutively to a number of drugs. Although the genetic evidence of the *plasmid cointegrate* group is not inconsistent with this idea, there are several theoretical considerations which speak against this "gene pick-up" model. Firstly, drug-resistance genes map at well-scattered sites around the *E. coli* and *Salmonella* chromosomes (207, 225). Incorporation of one genetic segment would favor subsequent crossing-over within this relatively extensive region of homology and therefore be likely to reduce integration at a different part of the chromosome to permit pick-up of a second segment. This model would be valid only if it were assumed that each of the drug-resistance markers is derived from one of a number of different host strains, none of which have extensive genetic homologies. Another consideration which speaks against the "gene pick-up" model is the fact that the modes of action of R-factor drug resistances are often functionally distinct from those of chromosomal resistance; R-factor resistances often involve antibiotic-inactivating enzymes (62, 63), whereas the corresponding chromosomal resistance comes about by a modification of ribosomal structure (245). More importantly, R-factor resistances function in a dominant fashion, indicating that in general the genes for sensitivity and resistance may be present simultaneously (233). This would not be necessary had the drug resistance arisen de novo on the chromosome of the haploid bacterium and furthermore, in many instances, chromosomal drug resistance (e.g., to streptomycin) is in fact known to be recessive (153).

One alternative to the "gene pick-up" model would propose that extrachromosomal DNA is commonly present in many bacterial species in the form of various independently replicating and autonomous replicons. Some of these replicons would take the form of transfer factors such as F. Others would take the form of noninfectious plasmids having colicin activity, as for example, ColEl. Still others might carry genes determining resistance to either antibiotics or heavy metals, or would be capable of mutation to this resistance. Since many noninfectious plasmids are conjugally transferred when coexisting in the same cell as one of a number of infectious plasmids (9, 43, 187, 221), the presence of an independent transfer factor and an independent noninfectious drug-resistance factor in the same cell would be likely to endow the cell with the properties of an R factor, i.e., infectious drug resistance (*see* Fig. 20). In some cases, genetic homologies, illegitimate pairing (82), or λ-integrase-like activity might permit subsequent integration of these replicons within each other to form composite replicons with the structure of plasmid cointegrates, but in the absence of cointegration the properties of such strains would be expected to be similar to those denoted for the group termed "plasmid aggregates." The model of the "plasmid aggregate" requires that the noninfectious plasmid should have the properties of a replicon, and it is not likely, therefore, to have arisen by the segregation of a chromosomal segment previously incorporated by gene pick-up. Thus, although the plasmid cointegrate model permits consideration of R-factor evolution via either chromosomal pick-

up or by mutation of extrachromosomal elements (followed by their mutual cointegration), the plasmid aggregate model would favor an extrachromosomal origin almost exclusively.

PHYSICAL COMPOSITION AND FORM

The probability that plasmids are composed of DNA was first concluded from the ability of F factors, R factors, and Col factors to be inactivated by the decay of radioactive ^{32}P incorporated in the host cell (151, 217, 243). However, the first direct demonstration of the physical nature of bacterial plasmids, laying the basis for future quantitative studies, came from the work of Marmur and colleagues (164). In this work, an F'*lac*$^+$ element originating in a strain of *E. coli* was transferred to a strain of *Serratia marcescens*, in which the lactose-fermenting activity associated with the F factor was stably inherited. Isolation of the DNA for example, ColE1. Still others might carry by analytical density-gradient ultracentrifugation in CsCl showed a major peak of DNA characteristic of the density of the *S. marcescens* chromosomal DNA (1.718 g/cm^3, corresponding to a base composition of 58% guanine plus cytosine [GC]), with a shoulder at a density corresponding to that of *E. coli* DNA (1.709 g/cm^3 = 50% GC). The shoulder was not visible in the parental strain of *S. marcescens* before transfer of the F factor. Later work demonstrated a similar F'*lac* transfer to strains of *Proteus* (in which the GC ratio and hence the density of the *Proteus* chromosomal DNA [1.699 g/cm^3 = 38% GC] were further removed from those of *E. coli* and of the F' factor), leading to the identification of the F' factor DNA as a separated satellite peak (77). The satellite peak was no longer present if the F' factor were cured by AO (*see* Fig. 3). Work with the F' sex factor was later extended to demonstrate the presence of similar satellite bands in DNA of *Proteus* strains to which one of several R factors had been transferred (74, 201). In some cases, a complex R-factor band was seen with peaks at two different densities. In these experiments prior to 1967, the technique used to isolate DNA from the donor strain led to its breakage into fragments with a maximum size about 10 million daltons (162). The double-peaked R satellite band, therefore, might have arisen either as a result of the independent transfer of two plasmids of distinct and different base composition or, alternatively, by the fragmentation of a larger plasmid greater than 10×10^6 daltons and of a heterogeneous base composition. Since F-factor DNA and λ DNA are known to be comprised of segments with different DNA base ratios (164, 219) as distinct from the more homogeneous base-ratio distributions among fragments of virulent phage and bacterial DNA species (163), no clear-cut distinction could be made between these two ideas.

The various configurations taken up by DNA molecules, which has formed the basis for much of the structural studies of bacterial plasmids, were previously well investigated and characterized by Sinsheimer and co-workers (27, 79) from studies with the double-stranded replicative form of the single-stranded phage, $\phi\chi174$, and by Vinograd and co-workers (229) in their experiments with polyoma virus. They have shown (Fig. 4) that DNA may take the form of (a) a linear, double-stranded (duplex) molecule which can close

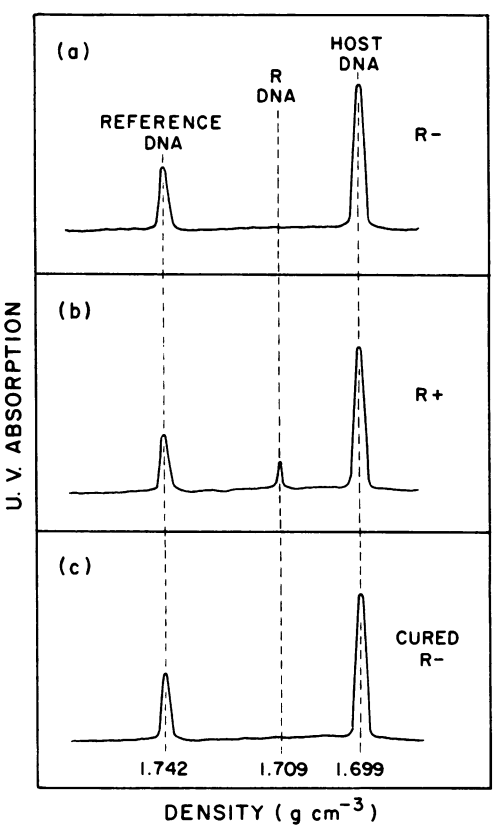

FIG. 3. *Diagrammatic representation of density tracings of ultraviolet adsorption photographs after analytical, density-gradient profiles of DNA from Proteus mirabilis, together with a reference DNA of Bacillus subtilis phage SP01, of density 1.742 g/cm^3. a, DNA from Proteus host at density 1.699 g/cm^3; b, DNA from the same host strain harboring an F' factor; c, DNA from the host strain after the F' factor has been cured by acridine orange.*

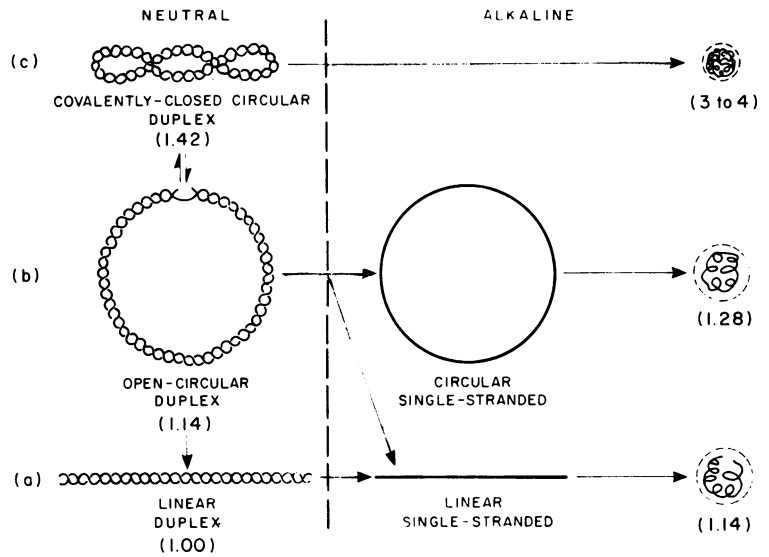

FIG. 4. *Diagrammatic representation of the different configurations of DNA molecules. The relative sedimentation coefficients of the different DNA molecules of polyoma virus in neutral and alkaline sucrose gradients are shown under the structures (193).*

upon itself to form a cyclic (circular) duplex molecule either (b) by joining one of the DNA strands, leaving the other with a break, or (c) through joining of both strands. Since both strands of this latter structure are covalently bonded, it is referred to as a covalently closed circular (CCC) duplex molecule. When isolated extracellularly, this molecule takes the form of a "supercoil," the DNA helix being twisted around itself. The circular form (b), with only one of its DNA strands joined, is termed somewhat confusingly an "open-circle" duplex because its conformation is open rather than supertwisted, a "nicked circle" because one of the two strands of the DNA duplex is broken or "nicked," or a "relaxed" circle because it is not supercoiled. The sedimentation values of these molecular forms of polyoma virus were carefully measured by Vinograd and colleagues in neutral solutions of either salt or sucrose to give the relative values shown in Fig. 4. The ratio of the sedimentation coefficients of the open circular to linear duplex (1.14) and that of the covalently closed to the open circular form (1.25) can be used in identifying each specific peak.

Under alkaline conditions, which lead to breakage of the hydrogen bonds between the two strands of a DNA duplex, the strands of the linear or open circular duplexes are separated, but the strands of the supercoil remain interlocked. Thus, when centrifuged under alkaline conditions, each structure forms a compact random coil with relative sedimentation coefficients as shown in Fig. 4, the covalently closed circular (CCC) form being a far more rapidly sedimenting molecule.

The sedimentation coefficients of similar forms of other molecules may be calculated from their molecular weights by use of formulas derived by the empirical fit of a number of experimental values.

That of the linear duplex is derived from

$$S^\circ_{20,w} = 2.8 + 0.00834\ M^{0.479}$$ (Svedberg) (87);

the open circular duplex varies as

$$S^\circ_{20,w} = 2.7 + 0.01759\ M^{0.445}$$ (Svedberg) (124);

and the covalently closed duplex varies as

$$S^\circ_{20,w} = 7.44 + 0.00243\ M^{0.58}$$ (Svedberg) (124).

Using these three formulas, the ratios of the sedimentation coefficients can be seen to vary with molecular weight (Fig. 5).

Vinograd and his co-workers also investigated the effect of the dye ethidium bromide on these structures (13, 192). Ethidium bromide is known to intercalate between adjacent base pairs of DNA, and in so doing the helix pitch is changed and the DNA duplex is extended, thus lowering its density. Intercalation of ethidium bromide occurs to the same extent

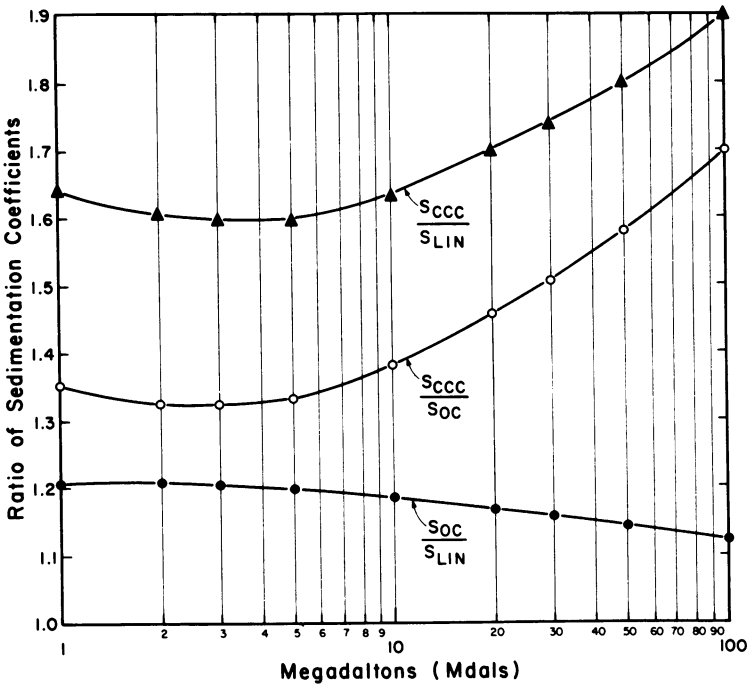

FIG. 5. *Ratios of sedimentation coefficients of configurational forms of duplex DNA as a function of molecular weight. The values S_{CCC}, S_{OC}, and S_{LIN}, respectively, represent the $S_{20,w}$ values in Svedberg units for the covalently closed circular, open (relaxed or nicked) circular, and linear duplex DNA molecules calculated from the empirical formulae. $S_{CCC} = 7.44 + 0.00243\ M^{0.58}$ (124); $S_{OC} = 2.7 + 0.01759\ M^{0.445}$ (124); $S_{LIN} = 2.8 + 0.00834\ M^{0.479}$ (87).*

in the open circular duplex or in the linear duplex, and this results in the same decrease in density (0.125 g/cm³) of both structures. In contrast, intercalation of ethidium bromide into CCC DNA is limited because of the restriction of the rotation of the two strands about each other, due to the absence of a free end of rotation. Thus, in the presence of ethidium bromide, the extension of CCC DNA is less than that of the other two duplex forms, and its density is thus lowered to a lesser extent (0.085 g/cm³). Hence in ethidium bromide, the CCC form is denser (0.125 − 0.085 = 0.04 g/cm³) and can be separated from the other two double-stranded forms even in the absence of any differences in base ratio.

METHODS OF ISOLATION

Differences from Host DNA Base Ratio

Host strains of *Proteus*, with a chromosomal GC content of 38% (density 1.698 g/cm³), have been frequently used for the isolation of plasmid DNA species, which have GC contents of between 45 and 55%, corresponding to densities from 1.704 to 1.718 g/cm³ (*see* Tables 4 and 6). Thus, plasmid DNA can be readily resolved from *Proteus* DNA and seen as a separated satellite peak or band after analytical density-gradient ultracentrifugation in cesium chloride solution (Fig. 6 and 7). Larger amounts of plasmid DNA may similarly be separated by preparative centrifugation, usually in a fixed angle rotor at 95,000 × *g* for 60 hr at 25 C. The contents of the centrifuge tube can then be fractionated by collecting drops through a hole punctured in the bottom. The DNA is assayed either by its ultraviolet adsorption at 260 nm or by measuring a tritium or ^{14}C-label incorporated in the DNA by the addition of radioactive thymidine (or thymine) to the culture during growth (Fig. 8). Further purification and enrichment can be achieved by column chromatography with methylated albumin kieselguhr (MAK), which fractionates by both molecular weight and base composition, lower GC DNA, and higher molecular weights eluting with increasing ionic strength (88). Plasmid DNA tends to elute with the first samples (73).

Early studies on the DNA of bacterial plas-

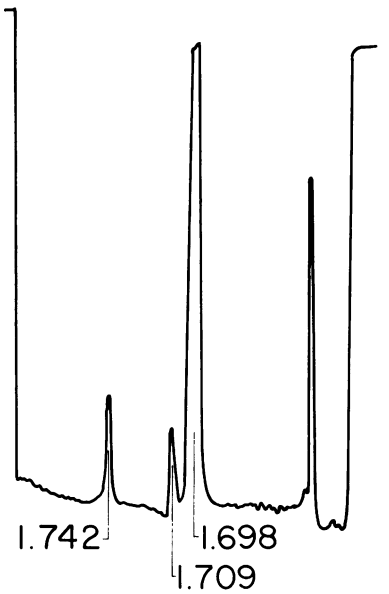

FIG. 6. *Microdensitometer tracing of ultraviolet adsorption photograph after analytical ultracentrifugation for 27 hr at 44,000 rev/min in a cesium chloride density gradient of DNA from a Proteus (R15)⁺ host strain (1.742 g/cm³ is the density of a reference DNA [Bacillus subtilis phage SP01 DNA]). The major peak at 1.698 g/cm³ represents the chromosomal DNA of the Proteus host strain (176).*

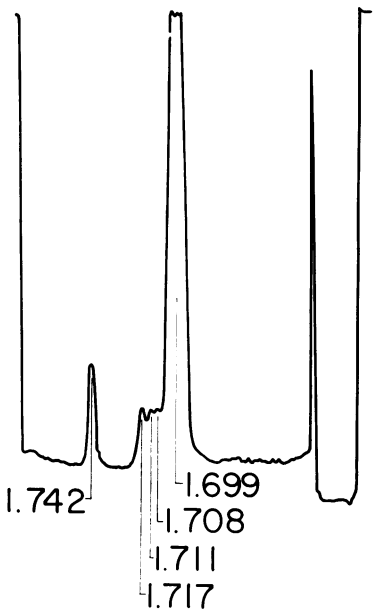

FIG. 7. *Microdensitometer tracing of ultraviolet adsorption photograph after analytical untracentrifugation for 27 hr at 44,000 rev/min in a cesium chloride density gradient of DNA from a Proteus (222)⁺ host strain (1.742 g/cm³ is the density of a reference DNA [Bacillus subtilis phage SP01 DNA]). The major peak at 1.699 g/cm³ represents the chromosomal DNA of the Proteus host strain (176).*

mids used the technique of Marmur (162) for DNA isolation which leads to its breakage into fragments of approximately 10 million molecular weight. A more gentle method of DNA extraction, applied by Helinski and co-workers (115), consists, first, in the formation of spheroplasts by the addition to the bacteria of a mixture of lysozyme, ribonuclease, and ethylenediaminetetraacetic acid (EDTA) in sucrose, followed by lysis with an ionic detergent, e.g., sodium lauryl (dodecyl) sulfate (SLS = SDS), after which protein is removed by extraction with carefully buffered phenol. After removal of phenol by dialysis, the DNA is centrifuged as above. Preparations adequate for configuration studies and molecular weight assays may be obtained from this improved method of DNA isolation by using centrifugation only, without recourse to column chromatography. Molecules of over 100×10^6 molecular weight have been isolated by this method (*see* Tables 3 and 4).

From Minicells

Several *E. coli* mutants have been identified as having an abnormal cell division that re-

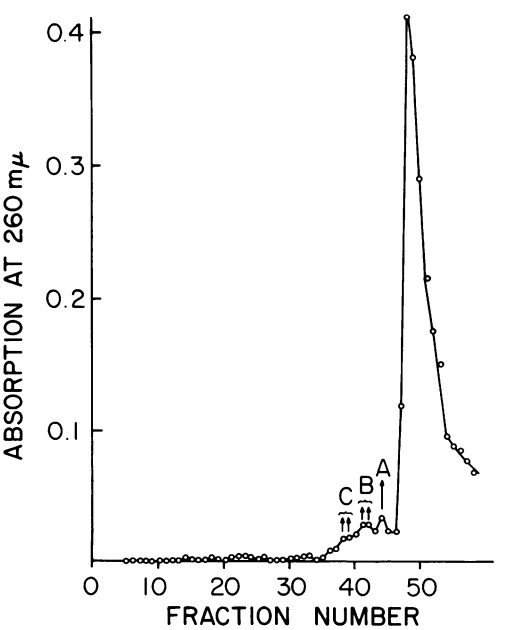

FIG. 8. *Ultraviolet absorption (260 nm) of fractions obtained after centrifugation of DNA from a Proteus (222)⁺ strain in cesium chloride in a preparative ultracentrifuge for 60 hr at 95,000 × g (176).*

sults in the segregation of progeny cells which do not contain any appreciable amounts of chromosomal DNA. One such mutant of *E. coli* K-12, P678-54, has been extensively investigated. Its DNA-less progeny, termed "minicells," are produced by a variety of growth conditions in a ratio to normal cells of up to 3:1. Minicells are about one-tenth the volume of normal cells and contain no detectable DNA and greatly reduced amounts of three presumably DNA-associated enzymes (ribonucleic acid [RNA] polymerase, DNA methylase, and the photoreactivating enzyme); however, near normal amounts of DNA polymerase I are present (3, 47). Minicells can be separated from normal-sized parental cells by differential centrifugation, followed by two successive sucrose-gradient centrifugations (3) or by growth in penicillin (155). The fraction of parental cells is thereby reduced to less than 1 to 10^6 minicells, which are concentrated as high as 10^{11} per ml. Minicells may be lysed by standard methods effective with normal cells. It has been shown that the DNA of many bacterial plasmids such as ColE1 (127, 129), ColB (195), R1 (157), R222 (128, 158), N3 (156, 157), and R64 (156, 157, 195) is segregated efficiently into minicells, almost all minicells harboring one or more plasmid copies. In contrast, F segregates very inefficiently to less than 1% of minicells (140). However, minicells derived from an F^- parental strain can act as efficient recipients for the conjugal transfer of plasmids from normal-sized donor cells. F and a variety of F′ factors can be transferred, although only a limited amount of chromosomal transfer occurs, and even DNA transfer of the F′ factor is limited, only shorter F′ factors being totally transferred (47). Minicells harboring sex factor plasmids have also been shown to be efficient donors (158, 195). Thus, the DNA of exconjugant or segregant minicells is almost entirely plasmid DNA and therefore provides an efficient biological separation of plasmid from chromosomal DNA.

Lysis with Nonionic Detergent (Brij 58)

Several methods of gentle lysis of bacterial cells have been developed, during which chromosomal DNA remains attached to a cellular component during low-speed centrifugation. Helinski and his co-workers (36) have applied one of these techniques using the nonionic detergent, polyoxyethylene cetyl ether (Brij 58; reference 96), to the isolation of plasmid DNA. They have found that, when cell spheroplasts prepared in the usual way in buffered sucrose and lysozyme are lysed by the addition of Brij 58 in the presence of sodium deoxycholate (DOC) and EDTA, much of the plasmid DNA remains in the supernatant fluid, even though the bulk (c. 95% of a radioactive thymidine label) of the chromosomal DNA continues to be sedimented by low-speed centrifugation. It may be concluded that, since a decrease in concentration of Mg^{2+} permits molecules of increasing size to be released (96), plasmid DNA might be released in the complete absence of Mg^{2+} when the membrane is sufficiently disorganized, although chromosomal DNA would remain associated with the membrane. Alternatively, since DOC dissociates membrane lipoprotein or lipopolysaccharide components (96), plasmid DNA may be bound by a DOC-sensitive attachment to the membrane. The supernatant fluids from such Brij-lysed preparations have been termed "cleared lysates" and have been extensively used for the separation of plasmid DNA. This technique has also been adapted for the isolation of plasmid DNA from *S. aureus* by using the enzyme lysostaphin in place of lysozyme (179).

Conjugal Transfer

A technique for specifically labeling plasmid DNA was developed by Freifelder and Freifelder using a system in which only those DNA molecules transferred to a recipient cell are able to incorporate radioactive label (90). The conjugal system uses an *E. coli* K-12 donor strain which cannot incorporate exogenous labeled thymine due to a genetic block, and a recipient which is prevented from replicating its own DNA due to a large number of lesions produced in the DNA by a prior heavy dose of ultraviolet light. During conjugation, the plasmid is transferred to the ultraviolet-killed recipient with near normal efficiency and can replicate in the irradiated cells. During this replication, it can take up labeled thymine, and its DNA can therefore be identified and measured in recipient cell lysates. A modification uses a thymidine kinase-deficient (*tdk*) mutation in the recipient strain, preventing incorporation of exogenous thymidine to less than 0.1% of that by the tdk^+ donor strain. Thus, specific labeling of DNA in the donor without inhibiting DNA synthesis in the recipient is possible, and the transfer of a labeled plasmid can be followed (228). The use of a recipient strain, carrying a mutation to resistance to phage T6, and a T6-sensitive donor strain allows selective lysis of donor cells, after which recipient cells can be lysed by lysozyme

and detergents in the usual manner.

Isolation of CCC Plasmid Molecules

Early experiments separating plasmid DNA by virtue of its difference in base ratio from host DNA (49, 115, 176, 197) had consistently indicated a proportion of molecules with "supercoiled" CCC structures. It is thus possible to apply techniques developed by Vinograd and others for the isolation of molecules of similar configuration from polyoma virus to studies of plasmids.

Cellulose nitrate adsorption. If a mixture of chromosomal and plasmid DNA is subjected to shearing forces, conditions can be chosen such that the chromosomal DNA can be fragmented, whereas plasmid DNA (because of its smaller size and CCC configuration [79]) is left intact. (Shearing may be accomplished by drawing the DNA through a capillary pipette [e.g., 0.1 ml] or fine-needled syringe [14] or by blending or vortexing [89].) If such a mixture of sheared chromosomal and plasmid DNA is briefly exposed either to high temperature or *p*H (>11.5), the hydrogen bonds between the strands of the DNA duplex are broken and the strands of the linear (chromosomal) DNA are separated (denatured), leaving the CCC plasmid DNA strands interlocked. [If the temperature is rapidly reduced, or if the alkalinity is maintained, the strands of linear DNA remain separated, although with this temperature reduction, the interlocked CCC strands can reform the duplex. If the *p*H is returned to neutrality, or if the preparation is allowed to cool down slowly, the double-stranded linear structure can be reformed (renaturation, reassociation, annealing).] Since single-stranded DNA will bind to nitrocellulose whereas double-stranded DNA will not (182), the single-stranded chromosomal DNA can be separated from the plasmid DNA duplex, either by passage through a nitrocellulose column (228) or by filtration through a cellulose nitrate membrane (250). This method has been used to separate a plasmid DNA that has a higher GC composition than its host and which would, even in the linear form, have been more stable to denaturation (250). It has also been used in early studies separating plasmid DNA from *S. aureus* (205).

Alkaline sucrose sedimentation. DNA released by lysis with lysozyme and detergent (SDS) is first sheared to degrade the chromosomal DNA into fragments small enough so as not to interfere with separation by centrifugation. After shearing, the degraded lysate is denatured in 0.3 M NaOH, layered on a 5 to 20% sucrose gradient containing 0.3 M NaOH, and sedimented to separate the CCC DNA as a peak which sediments three to four times more rapidly than linear or open circular (OC) DNA (*see* Fig. 4).

Ethidium bromide-cesium chloride centrifugation. A crude cell lysate made by the action of detergent on spheroplasts is sheared as above and is added directly to a mixture of cesium chloride and ethidium bromide. Since SDS is insoluble in cesium chloride, the detergent used is sodium sarcosinate (Sarkosyl) (14). The mixture is immediately centrifuged, resulting in the separation of the CCC DNA as a denser band below the less dense open circular or linear DNA, the bulk of which is chromosomal DNA (*see* Fig. 9).

METHODS OF MOLECULAR WEIGHT DETERMINATION

Sedimentation Analysis

The molecular weights of plasmid DNA isolated by methods described above can be estimated from the sedimentation coefficients of

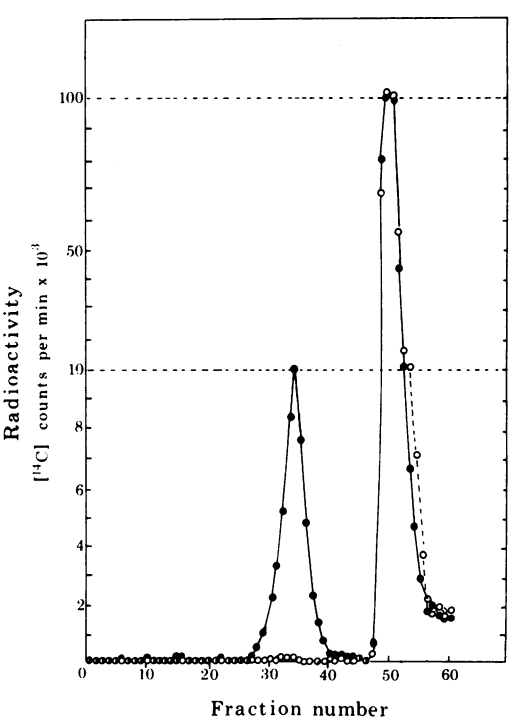

FIG. 9. *Radioactivity of fractions obtained after ultracentrifugation in ethidium bromide-CsCl solution of crude lysates of E. coli strains grown in radioactive thymidine. From an R⁻ E. coli host* (○); *from the same host carrying an R factor* (●) (*177*).

the various configurational forms in neutral sucrose gradients. A mixture of plasmid DNA in the CCC, OC, and linear double-stranded forms would sediment in neutral sucrose with sedimentation coefficients in the ratios shown in Fig. 5. The separation of a CCC peak characteristic of plasmid DNA can be achieved in the presence of chromosomal or nonplasmid linear fragments. The OC form can also usually be identified as a sharp peak, but linear plasmid DNA is usually poorly resolved and is not normally isolated by most techniques. In practice, therefore, two peaks (CCC and OC) with relative sedimentation coefficients of 1.33 to 1.7:1 (depending on molecular weight of plasmid, Fig. 5) are usually observed (Fig. 10). (In those cases where deviation from the expected ratio is observed, the quantitation below must obviously be viewed with caution.) The identity of the CCC and OC peaks can be confirmed by taking advantage of the fact that a single scission or nick in just one of the strands of a CCC molecule produces an OC molecule (Fig. 4). This may be done by one of several methods, including limiting deoxyribonuclease treatment (76, 79, 128, 229) or X-irradiation (85), and leads to a decrease in the size of the CCC peak and a corresponding increase in the size of the OC peak which are thereby defined. The absolute value of the sedimentation coefficient for the OC peak may be determined by cosedimentation of a carefully purified DNA standard of known sedimentation coefficient and by application of the formula, $S1/S2 = D1/D2$ (26), where $S1$, $S2$ are the sedimentation coefficients and $D1$, $D2$ the distances of the peaks from the meniscus of the OC and standard peaks, respectively. The sedimentation coefficient ($S3$) of the linear form of the plasmid is then approximated by dividing the calculated value of $S1$ by the value expected from the ratio OC:linear at the rough value of molecular weight (Fig. 5). If the molecular weight of the standard DNA is known, the molecular weight of the linear form of the plasmid may now be estimated according to the formula $S2/S3 = (M2/M3)^{0.38}$ (26), where $M2$, $M3$ are the molecular weights of standard and linear form, respectively. Exponent 0.38 is an empirical figure derived from a number of carefully measured values of M, and is a revision (87) of the previous figure of 0.35 derived by Burgi and Hershey (26) and used in most calculations referred to in this review.

Loss of Supercoil Structure by Irradiation

As noted above, a single nick in just one of the strands of the duplex is sufficient to convert a CCC into an OC molecule. Freifelder applied this technique to obtain some of the earliest measurements of the molecular weights of the CCC molecules of F and F′ factors by measuring the relative losses of CCC DNA and corresponding gains in OC DNA in a series of DNA samples exposed to increasing doses of X-irradiation (86). The logarithm of the fraction of surviving CCC molecules is a linear function of the irradiation dose, the slope of the survival curve being proportional to the molecular weight. Freifelder estimated

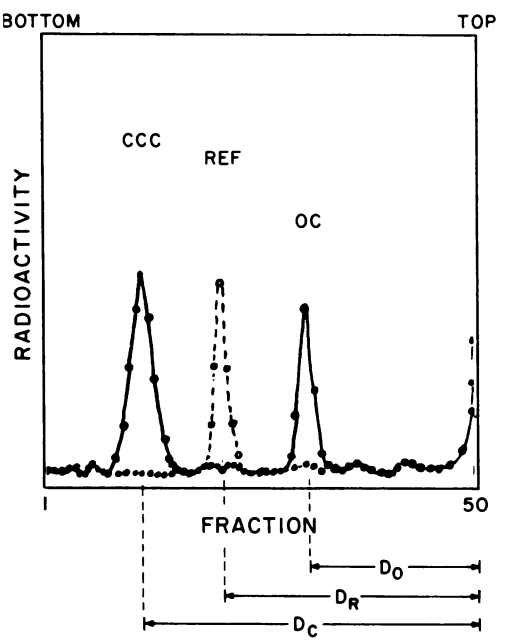

FIG. 10. *Diagrammatic representation of a neutral sucrose gradient profile. The radioactive DNA preparation, together with a differentially labeled reference DNA of known sedimentation coefficient and molecular weight, is added to the top of a 5 to 20% sucrose gradient in a centrifuge tube. After centrifugation for 8 hr at 75,000 × g, 50 equal-volume fractions taken through a hole punctured in the bottom of the tube were assayed for radioactivity. The reference DNA is distinguished by its differential radioactivity, and the covalently closed circular (CCC) and the open circular (OC) peaks are recognized either by their ratio of sedimentation coefficients or by a parallel experiment where the unknown DNA is exposed to limiting deoxyribonuclease action resulting in a diminution of the CCC and an increase in the OC peaks. The sedimentation coefficients of the CCC and OC peaks can be calculated by substitution of the distances (DO, DR, DC) of each peak from the top of the gradient into the formula D1/D2 = S1/S2.*

the molecular weights of a number of F elements in this way by comparing the rates of survival of their CCC molecules to that of λ CCC DNA, whose molecular weight is accurately known (61).

Reassociation Kinetics

If a preparation of DNA molecules is sheared to produce fragments of about 5×10^6 molecular weight, heat-denatured to separate the two strands of the double helix, and then slowly cooled, each single-stranded fragment will find and reassociate with its homologous complementary strand to form a double-stranded fragment. The time taken for this reassociation will depend upon the probability of a single-stranded fragment finding its homologue. This in turn will depend upon the concentration of homologues. Clearly, fragments of large DNA genomes will contain less homologues for the same total DNA concentration than those of small DNA genomes, and reassociation will be slower. Thus, measurements of rates of reassociation give a measure of genome size (24). Since the absorption of ultraviolet light by double-stranded DNA is less than that by single-stranded DNA, the change from the single- to double-stranded form by reassociation can be followed by ultraviolet absorption measurement. The fraction of reassociated molecules is then plotted against the logarithm of the product of the initial DNA concentration (C_0) and the time (t), (i.e., C_0t) measured in moles of nucleotide per liter second. The 50% C_0t value being proportional to the original molecular weight is about 8 for molecules of *E. coli* (2.5×10^9 molecular weight) and about 0.1 for a plasmid of 40×10^6 molecular weight (24).

Electron Microscopy

If DNA is allowed to diffuse to an air/water interface at which there is a monolayer of a protein such as cytochrome *c*, the acidic groups of the nucleic acid molecules and the basic groups of the protein molecules interact in such a way as to adsorb the long, thin DNA molecule to the surface of the liquid (143). If a sample is then removed from the protein monolayer film and examined by electron microscopy, the DNA is found to be extended in individual molecules (143, 150), in contrast to the usual aggregated and oriented structures (148; *see* Fig. 11). Plasmid DNA isolated by a variety of methods consists largely of circular double-stranded molecules. Many of these molecules show the expected supertwisted structure, but some will always have an open circular configuration (Fig. 12a). When the DNA molecules seen in the electron microscope are photographed and projected on a screen, the contours of the open circular molecules can be traced and their lengths accurately measured by a map measurer (149). Most plasmid DNA is found to contain molecules of one size. (A particular advantage of a circular molecular species is that fragmentation or degradation of the DNA results in loss of the circularity, and thus all circular molecules are by nature intact.) From a knowledge of the magnification, the physical length of the molecule (l) can be determined with a reproducibility of about ±2% (147). If the value of the ratio of molecular weight to length (M/l) is known for the conditions of electron microscopy preparation, the molecular weight of the DNA concerned can be derived. The assumption is made that M/l is independent of the base composition of the DNA; since the base compositions of most plasmids do not vary more than about 45% to 55% GC (Table 4), this assumption is not unreasonable. The most recent calibration for preparations in ionic strength of 0.3 M is $(2.07 \pm 0.04) \times 10^6$ daltons per μm (147). (In an attempt to relate molecular weight determinations from a number of laboratories, this conversion figure will be used for all molecular weight calculations, even though many published data have used other M/l values.) Alternatively, standard DNA molecules of uniform size and known molecular weight (e.g., λ DNA) may be added before sampling and serve for calculating M (227).

SIZE AND CONFIGURATION

F and F′ Sex Factors

Freifelder isolated the DNA of an F′*lac* plasmid specifically labeled after conjugal transfer and first confirmed it as being that of the plasmid by its ability to bind on an agargel column to *Proteus* PM1(F′*lac*)$^+$ DNA without showing binding to DNA of the parental *Proteus* PM1 strain, not carrying the F′*lac* factor. The sedimentation coefficient of the transferred DNA, which formed a broad band on sucrose sedimentation and was therefore assumed to consist of both OC and linear molecules, was measured by cosedimentation with a pure nonfragmented preparation of T7 DNA. Calculation by application of the formula above gave a molecular weight of 55 ± 8

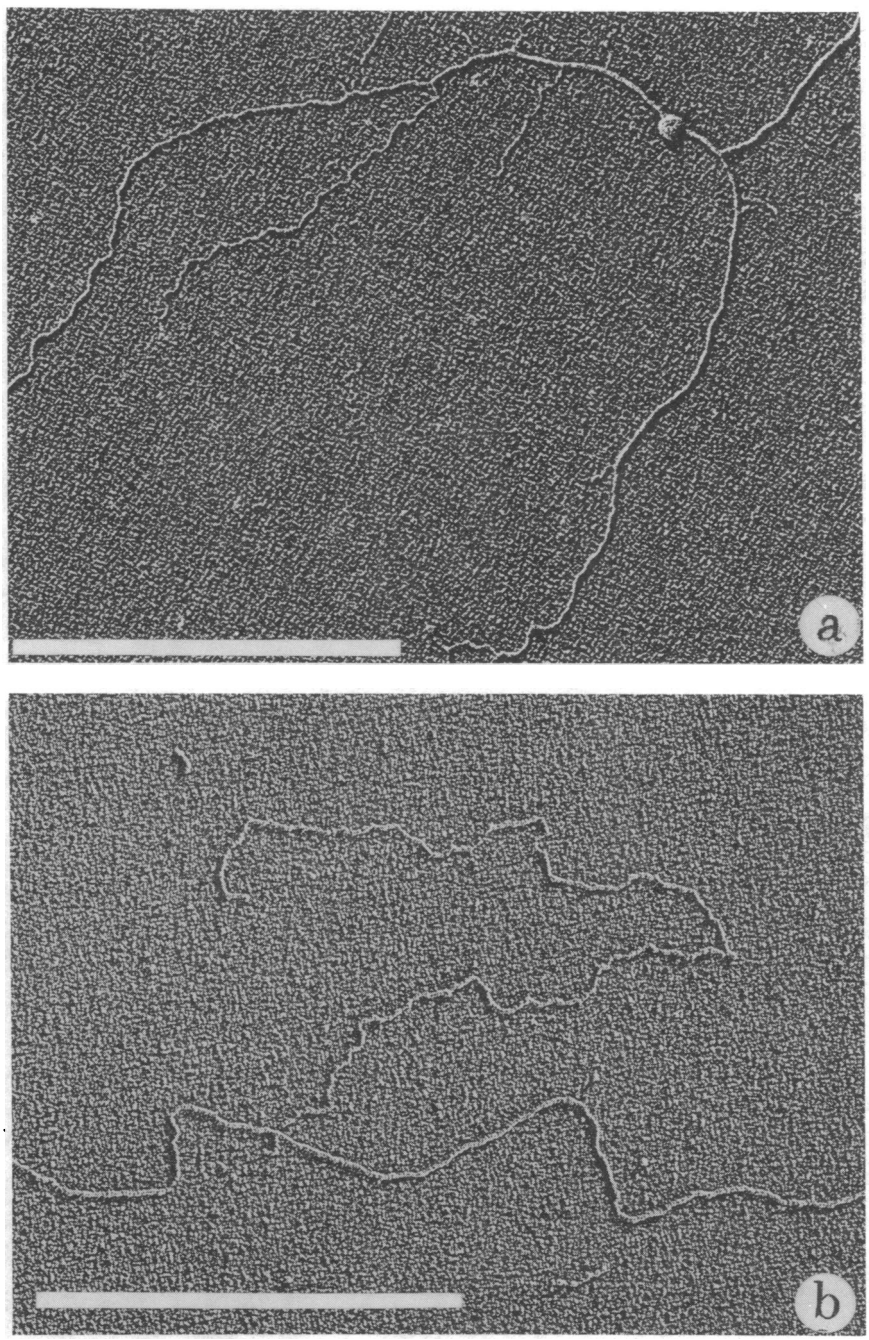

FIG. 11. *a, Electron micrograph of DNA dried onto a specimen grid and shadowed with platinum. Lateral aggregation and preferential orientation in one direction are typical drying artifacts. The bar represents 1 μm. ×51,000 (148). b, Electron micrograph of double- and single-stranded DNA prepared on the same grid by the diffusion method (148). Single-stranded DNA has less contrast and is more kinked. Bar indicates 1 μm. ×57,000 (148). (Electron micrographs kindly provided by D. Lang.)*

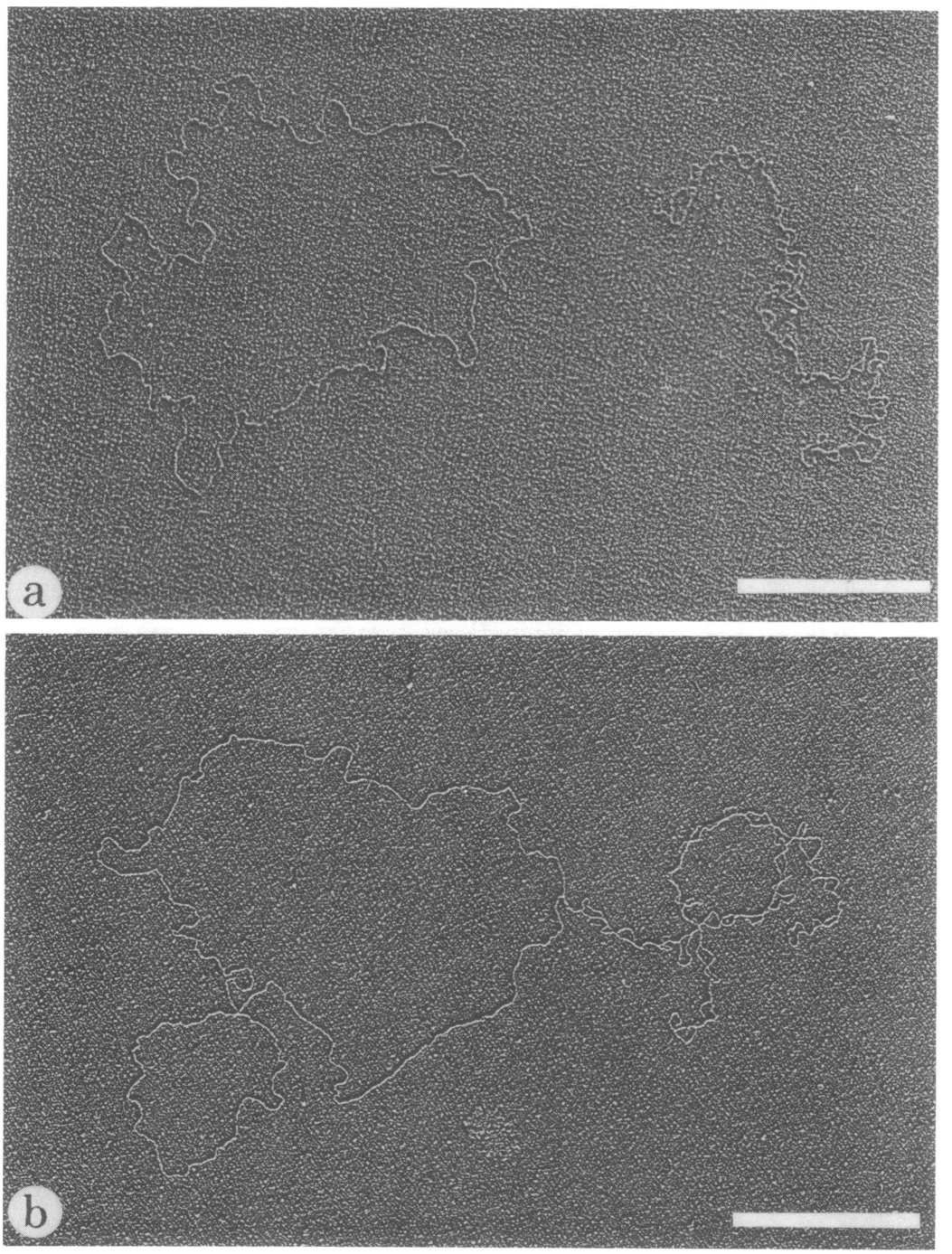

FIG. 12. *Electron micrographs prepared by the diffusion method of R-factor DNA taken from satellite band after dye-buoyant density centrifugation of E. coli R^+ cultures. a, Nicked (open) circular and supertwisted forms of R6K (12.8 μm) are shown. Bar indicates 1 μm. × 27,000 (145). b, Electron micrograph of R6K dimer. Catenated dimer consisting of one supertwisted monomer interlocked with nicked circular monomer. Bar indicates 1 μm. × 27,600 (145).*

Mdal [molecular weights will be expressed in daltons $\times 10^6$ (megadaltons ≡ Mdal)] for F'*lac* and a preliminary estimation of the F factor of 35 ± 7 Mdal (91). Later experiments (85) exploited the separation of CCC DNA of F' elements by alkaline sucrose and ethidium bromide-cesium chloride sedimentation. X-irradiation inactivation curves of such CCC DNA using λ CCC DNA as a standard resulted in the estimation of the molecular weights of a series of F' elements (*see* Table 2).

Bazaral and Helinski later employed Sarkosyl lysis, followed by ethidium bromide-cesium chloride centrifugation to isolate F-DNA which sedimented in sucrose gradients in two peaks at 80*S* and 48*S* (15). Assuming that these two peaks corresponded, respectively, to the CCC and OC forms (ratio CCC/OC = 1.66), a molecular weight of 75 Mdal was estimated, using the 48*S* value. Electron microscopy of F-DNA isolated by the same procedure by several workers and measured by contour length has led to a molecular weight of 61 to 64 Mdal (44, 144, 188). F-DNA also isolated as a CCC molecule after conjugal transfer and measured by electron microscopy using an internal λ DNA standard was calculated as 62 Mdal (227)

The electron microscopic measurements of the contour length of F from at least five sources in four laboratories have led to remarkably similar results of contour length measurements of 30.8 to 31.7 μm, conforming to a molecular weight of 64 Mdal, if an M/l ratio of 2.07 Mdal per μm is assumed (Table 2).

F'*lac* DNA (165) has also been isolated by a similar procedure and measured by electron microscopy.

Colicinogenic Plasmids

DeWitt and Helinski (67) showed that DNA from a strain of *Proteus* harboring the ColE1 factor had a satellite band at a density of 1.710 g/cm^3 which was not present in the parental strain. Other attempts to isolate Col factor DNA from *Proteus* have been unsuccessful, probably owing to the instability of the plasmids in *Proteus* and the lack of a selective mechanism. (It has recently been claimed that I-like R factors [but not F-like or other non-I-like fi$^-$ factors] cannot be transferred to *Proteus* strains [59].) The ColE1 plasmid from *P. mirabilis* was later isolated and identified by a technique including alkaline denaturation of the DNA. This was followed by CsCl centrifugation and electron microscopy of the satellite DNA to show circular molecules of 2.3 μm and 4.7 μm in a 2:1 ratio (and a number of supertwisted CCC molecules) corresponding to molecular weights of 4.5 and 9.2 Mdal for the monomeric and dimeric forms of ColE1, respectively (198). (Using the corrected value of M/l, the monomeric form corresponds to 4.8 Mdal [Table 3.]) Separation of ColE1 from *E. coli* by the ethidium bromide method led to the isolation of the DNA as a 23*S* CCC and a 17*S* OC form corresponding to a molecular weight of 4.8 Mdal as previously found. In contrast to *Proteus*, no multimeric forms were found in *E. coli*. Application of a similar technique to ColE2 and ColE3 resulted in sedimentation peaks at 25*S* leading to an estimation of molecular weights of approximately 5 Mdal for each of these factors (14). DNA from ColE1$^+$ minicells was found by Inselburg (127) to sediment as a 24*S* peak, concluded to be formed of CCC molecules, since the kinetics of treatment with pancreatic deoxyribonuclease gave rise to its exponential conversion to an 18*S* (OC) form (127). Separation of ColE1 mole-

TABLE 2. *Molecular weights of F sex factors*

Plasmid	Method of molecular wt determination[a]	Molecular wt (Mdal)[b]		Reference
		Q	C	
F	X	45	42	85
	S	35	34	91
	S	75		15
	EM	61	66	144
	EM	64	64	44[c]
	EM	62	64	227
	EM		64	188
F'-Lac[d]	X	74	69	85
	S	55	52	91
	EM	72	78	165
F'-Gal	X	51	48	85
F'-Galλatt	X	72	67	85

[a] S, Neutral sucrose sedimentation; X, X-ray inactivation; EM, contour length measurement by electron microscopy.

[b] Megadaltons (Mdal) equivalent to atomic mass units $\times 10^6$; Q, value quoted in reference; C, corrected value; using 0.38 (87) for sedimentation index in place of 0.35 (26); using 30.8 Mdal for molecular weight of λ standard (61) in place of 33 Mdal used in X-irradiation data (85) or 30 Mdal used as electron microscopy standard (227), and assuming M/l ratio for double-stranded DNA of 2.07 Mdal per μm (147).

[c] Including unpublished data by author in collaboration with M. Mitani using F from *E. coli* K-12 strain W1485F$^-$; based on 32 molecules of 30.7 ± 0.2 μm, and 23 of 31.0 ± 0.5 μm (two independent isolates).

[d] F-Lac from Jacob strain 200P.

TABLE 3. *Molecular weights of colicinogenic factors*

Plasmid	Method of molecular wt determination[a]	Molecular wt (Mdal)[b]		Reference
		Q	C	
ColE1	EM	4.5	4.8	197
	EM		4.8	129
ColE2	S	5.0	6.0	14
ColE3	S	5.0	6.0	14
ColIb	S	61.5	53.5	38
	EM	68	68	44[c]
ColB2	EM	70	70	44[d]
ColV2	EM	94	94	44[e]
FVBtc[f]	EM	107	113	115

[a] S, Neutral sucrose sedimentation; EM, contour length measurement by electron microscopy.

[b] Q, quoted molecular weight in reference; C, corrected molecular weight: electron microscopy by use of 2.07 Mdal per μm (147) in place of 1.96 Mdal per μm (161).

[c] Including unpublished data by author in collaboration with M. Mitani based on measurement of 32 molecules of 31.8 $\pm$ 0.4 μm, 13 of 33.8 $\pm$ 0.7 μm, and 6 of 35.5 $\pm$ 0.6 μm.

[d] Including unpublished data by author in collaboration with M. Mitani based on measurement of 29 molecules of 33.9 $\pm$ 0.5 μm.

[e] Including unpublished data by author in collaboration with M. Mitani based on measurement of 4 molecules of 47.0 $\pm$ 0.7 μm, 7 of 46.1 $\pm$ 0.3 μm, and 4 of 43.0 $\pm$ 2.5 μm.

[f] Fredericq F·ColV·ColB·*trp*·*cys* plasmid (84).

cules replicating in minicells was achieved by their incorporation of a heavy (5-bromouracil) label, and their subsequent examination by electron microscopy led to an average contour length measurement of 2.31 $\pm$ 0.06 μm (4.8 Mdal; reference 129), corresponding very closely to the previous measurements above. The large F·ColV·ColB·*trp*·*cys* plasmid of Fredericq (84) was isolated from a *Proteus* host strain using lysozyme, SDS, and phenol extraction followed by density gradient centrifugation in CsCl (115). A satellite band at 1.710 g/cm^3 was identified from which electron micrographs showed open and supertwisted molecules with contour length of 54.5 $\pm$ 1.7 μm corresponding to a molecular weight of 107 (corrected 113) Mdal. Dye-buoyant density gradient centrifugation of DNA from *E. coli* strains harboring one of a number of colicin factors has also been used to isolate a number of different Col-factor DNA species in this laboratory, the measurement of contour length by electron microscopy resulting in molecular weight determinations also summarized in Table 3.

Drug-Resistance Factors

Monomolecular factors. Wild-type R factors have been transferred to strains of *Proteus* and *E. coli* (including minicell-producing strains) from which the DNA has been isolated and plasmid DNA separated by a variety of methods, including base-ratio differences, ethidium bromide-cesium chloride centrifugation, and alkaline sucrose sedimentation. The DNA was found in all instances to be represented by circular molecules, both CCC and OC, the contour lengths of the OC molecules being usually of uniform size. The densities, methods of isolation, molecular weight determination, and the sizes of these R factor molecules are summarized in Table 4 and range in densities from 1.704 to 1.711 g/cm^3 and in sizes from 26 to 78 million molecular weight.

Segregant R factors have been derived by transfer of wild-type factors to strains of *Salmonella typhimurium* in which it has been shown (240) that many R factors are unstable and tend to lose some, and occasionally all, of their drug resistances. Two such segregant types of 222/R4 (conferring resistance to sulfonamides [Su^r], streptomycin [Sm^r], chloramphenicol [Cm^r], and tetracycline [Tc^r]) were isolated, one, 222/R3(N), having lost Tc^r and the other, 222/R1, having lost Su^r, Sm^r, and Cm^r (177). In all, only one R3 segregant type was isolated, but five independent R1 segregants were isolated and designated R1A through R1E. The factors were transferred to *E. coli* hosts and DNA was isolated. All segregants showed genetic loss to have been accompanied by physical loss of DNA, since DNA of segregant factors was shorter in contour length than the parental 222/R4 factor DNA (Table 5).

Among the R1 segregants, two groups were found, R1A having lost more DNA than R1B, and R1C, D, and E resembling R1B in the loss of similar amounts of DNA, assumed to be due to the same genetic deletion. R1A and R1B were selected as typical of these two groups. Molecular recombination between these segregants was demonstrated in *E. coli* by selecting from a mixed culture of host strains (one carrying 222/R1 and the other, 222/R3), "recombinant" strains carrying all four drug resistances. (Recombination between R factors has previously been demonstrated genetically [108] and has been shown to depend on the *rec* system of the host [81].) A number of colonies were isolated, all of which were stable and could transfer concommitantly all four drug resistances. Isolation of DNA from these

"recombinant" R factor strains indicated that molecular recombination had taken place, since the DNA of the "recombinant" R factor comprised a unimolecular species of size equivalent to that of parental 222/R4 from which the two segregant R factors were derived (Table 5 and Fig. 13).

Derivative R factors retaining transfer factor properties, which have lost all drug resistance markers (and therefore inferred to be the sex-factor element [RTF] responsible for the transfer of drug resistance), have been isolated from factors R1 and R6. That of R1 was shown to have a density of 1.709 g/cm^3 (51, 103) and sedimented in sucrose with peaks of 64*S* and 44*S*; the linear monomer was therefore calculated as 39*S*, equivalent to 50 Mdal (216). The RTF from R6 also sedimented at 1.709 g/cm^3 and, when isolated by ethidium bromide and measured by electron microscopy, its contour length was 26 to 34 μm, the mean value of 31 μm corresponding to a molecular weight of 63 Mdal (50).

Staphylococcal plasmids have recently been subjected to a number of physical studies by Novick and co-workers (179). Using a method of alkaline denaturation and nitrocellulose chromatography, the DNA of a wild-type penicillinase plasmid (PI) which also carries a number of other genes (*see* Fig. 2), and that of two of its segregants showing genetic deletions, were shown to be circular molecules of contour length 9.4 μm, 7.3 μm, and 8.1 μm, respectively (205). Thus, the PI penicillinase plasmid is a unimolecular species of molecular weight about 19 Mdal and undergoes deletions to produce segregants of about 17 and 15 Mdal. Two further independent *S. aureus* plasmids have recently been examined by an adaptation of the "cleared lysate" method with Brij 58 (36) and have led to contour length measurements of 1.4 μm for a plasmid (*tet*) controlling tetracycline resistance and 1.6 μm for another plasmid (*cml*) controlling chloramphenicol resistance. These two plasmids thus differ from the penicillinase plasmid PI not only in that they control only one type of resistance (Tc and Cm, respectively), but they are also much smaller, with molecular weights of 2.9 Mdal (*tet*) and 3.1 Mdal (*cml*) (179).

Unstable R factors. The DNA species of a number of R factors have been shown to be comprised of more than one molecular species. When DNA of the R1 and R6 factors was iso-

TABLE 4. *Molecular weights of monomolecular R factors*

Plasmid	fi[a]	Isolation[b]	Density[c] (g/cm^3)	GC[d] ratio	Method of molecular wt[e]	Molecular wt (Mdal)[f]		Reference
						Q	C	
R15	−	BR	1.709	49	EM	35	38[g]	176
		EB	1.708	48	EM	46	46	177
R6K	−	EB	1.704	45	EM	26	26	145
R28K	−	EB	1.710	50	EM	44	44	145
R64	−	CT			EM	76	78[h]	227
R538	+	CT			EM	49	50[h]	227
222/R4	+	EB	1.710	50	EM	70	70	177
		mini			EM	62	64[g]	128
222/R3W	+	EB	1.710	50	EM	69	69	177
R1	+	EB	1.710	50	S	63	59[i]	49
		CT			EM	65	68[g]	50
					S	65	61[i]	215
R6	+	EB	1.711	51	EM	64	67[g]	50
					S	64	60[i]	50

[a] Type of R factor indicated by fi group (239). All fi$^-$ factors are F-like; some fi$^-$ factors are I-like; others are not I-like (58).

[b] BR, By base ratio difference from *Proteus* host; EB, by ethidium bromide-cesium chloride from *E. coli*; CT, by conjugal transfer method in *E. coli*; mini, by segregation into *E. coli* minicells.

[c] Measured to an *E. coli* DNA standard of 1.710 g/cm^3 (164).

[d] Conversion from (210).

[e] S, Neutral sucrose sedimentation; EM, contour length measurement by electron microscopy.

[f] Q, Quoted molecular weight in reference; C, corrected molecular weight.

[g] By use of M/l factor of 2.07 Mdal/μm (147).

[h] By use of corrected λ MW of 30.8 (61).

[i] S value by use of sedimentation index of 0.38 (87).

TABLE 5. *Contour length measurements and molecular weights of R factor 222, its segregants, and its recombinants in E. coli (177)*

Plasmid	Contour length ± SSD[a] (μm)	No. of molecules measured	Mean length (μm)	Molecular wt (Mdal)
Parental				
222/R4	33.5 ± 0.5	21	33.6	70
	33.8 ± 0.7	12		
	33.8 ± 0.4	14		
Segregant				
222/R3N	29.5 ± 0.4	33	30.3	63
	30.5 ± 0.4	22		
	31.5 ± 0.8	17		
222/R1A	22.3 ± 0.8	25	22.3	46
222/R1B	25.9 ± 0.3	30	25.6	53
222/R1C	25.7 ± 0.3	35		
222/R1D	25.4 ± 0.3	28		
222/R1E	25.8 ± 0.3	20		
Recombinant				
R1AxR3N-1	32.6 ± 0.9	29	33.5	69
-2	34.3 ± 0.3	17		
-3	33.3 ± 0.5	20		
-4	33.9 ± 0.4	22		
R1BxR3N-1	32.9 ± 0.7	29	33.2	69
-2	34.1 ± 0.4	24		

[a] SSD, sample standard deviation.

lated from *E. coli*, in addition to the molecules of 60 to 70 Mdal found as a majority species (Table 4), a minority of smaller molecules was found (49, 50). DNA isolated from host strains of *Proteus mirabilis* carrying either of these two factors (50) contained three sizes of molecules with different densities (Table 6). Very similar results had previously been reported for the independent R factor 222/R3W (*see also* Fig. 14). From the experiments with 222, it was concluded that, since the sum of the sizes of the two smaller molecular species was equal to that of the largest species (which also had a density intermediate to the two smaller ones), that the two smaller molecules had resulted from segregation from the larger (i.e., composite) molecules in the host strains of *Proteus* (176). This composite molecule was concluded to be equivalent to the single molecular species later found in *E. coli* of size 33.5 μm (69 Mdal) (177 and Table 4; *see* Fig. 15). Similar conclusions were later drawn for the other factors R1 and R6 which, like 222, are also fi^+ factors (52, 215). It is interesting to note that, in spite of the similarity of these segregation patterns in *Proteus* and particularly in the molecular sizes of all three R factors and their segregants, their origins are distinct. Factor 222 (234), isolated in Japan (174), has also been termed NR1 (201) and R100 (72) by different workers and carries resistances to Su, Sm, Cm, and Tc. R1 originated in the United Kingdom (8) and determines resistance to Su, Sm, Cm, Km, and Ap, whereas R6, isolated in Switzerland (152), carries Su, Sm, Cm, Tc, Km, and Nm resistance. Homology studies of these geographically and phenotypically distinct R factors examining the DNA: DNA heteroduplex formation by electron microscopy would obviously be of interest, since it seems possible that at least the same transfer factor moiety may be involved. (A preliminary report [P. A. Sharp, N. Davidson, and S. N. Cohen, 1971, Fed. Proc., 1054/8] shows homology of ColV2, R1 and R6 with the same region of F. R1 and R6 show extensive homologies over their total length.) It has been suggested that *E. coli* or *Enterobacteriaceae* may be the normal hosts of these R factors (i.e., in which their evolution by the accretion of extrachromosomal drug resistance plasmids to a transfer factor may have occurred), where the stable composite form is preserved. *Proteus* may be considered as a foreign host in which the R factor breaks down to its evolutionary component molecules.

Multimolecular factors. Another system of R factors recently studied, the ΔSAT system of Anderson (7), shows that even in *E. coli* hosts some R factors may be comprised of (stable) multimolecular species. From an original strain carrying infectious resistance to streptomycin, ampicillin, and tetracycline, three segregant strains were isolated by Anderson and co-workers: one termed Δ^+S^+ carries infectious resistance to streptomycin, another referred to as Δ^+A^+ transmits infectious resistance to ampicillin, and a third, $(\Delta\text{-}T)^+$, shows infectious resistance to tetracycline (Fig. 16). From host cells carrying the first two factors, further segregants were isolated. The Δ^+S^+ strain gave rise to two segregant strains: one carried noninfectious resistance to streptomycin (termed S), and another was not drug resistant but carried a transfer factor (Δ), shown by virtue of the fact that, when grown in mixed culture with the S^+ strain, it could mobilize the transfer of streptomycin resistance to a recipient strain. From the Δ^+A^+ strain, two similar segregant types were isolated, one carrying the transfer factor Δ and the other with noninfectious ampicillin resistance, A. From $(\Delta\text{-}T)^+$ strains, no further segregants were isolated (5, 7, 9, 11). DNA was separated from each of the

plasmid-containing strains by Sarkosyl lysis followed by ethidium bromide-cesium chloride density centrifugation and in each case was composed of a mixture of CCC and OC molecules. The contour lengths of a number of OC molecules were measured as shown in Table 7 (168a). The DNA from a strain carrying Δ comprises molecules of one size only (29.1 μm contour length, equivalent to 60 Mdal). From a strain carrying S, again a monomolecular species was isolated of approximately one-tenth the size of Δ, and of molecular weight estimated therefore as 6 Mdal. In Δ^+S^+ strains, a bimodal distribution of molecules was found, corresponding in sizes to the molecules found in Δ^+ and S^+ strains, respectively. Similarly, DNA extracted from Δ^+A^+ strains formed a similar bimodal distribution of molecules. In contrast, $(\Delta\text{-}T)^+$ strains comprised a monomolecular species of 32.3 μm contour length (67 Mdal).

From a comparison of length measurements of small molecules inferred to be S, isolated from Δ^+S^+ strains, we concluded that there is no real difference in size from those isolated from S^+ hosts. Moreover, the size distribution of large molecules from both Δ^+A^+ and Δ^+S^+ strains corresponds with that of molecules found in DNA of Δ^+ strains, except for a small number of molecules of the size one might expect of a recombinant molecule between Δ and either A or S. However, this number is less than 5% of the total number of large molecules. It was concluded that in Δ^+S^+ strains the majority of molecules act as independent Δ and S molecules, and little if any recombination has taken place, although recombination cannot be completely excluded. A similar conclusion was drawn from the ΔA data. In contrast, from data with Δ-T DNA, it was concluded that this plasmid represents a stable union of Δ and T (a tetracycline-resistant determinant) and takes the form of a monomolecular species larger than Δ by an amount about the size of A or S (168a). In this way, the physical data supported the conclu-

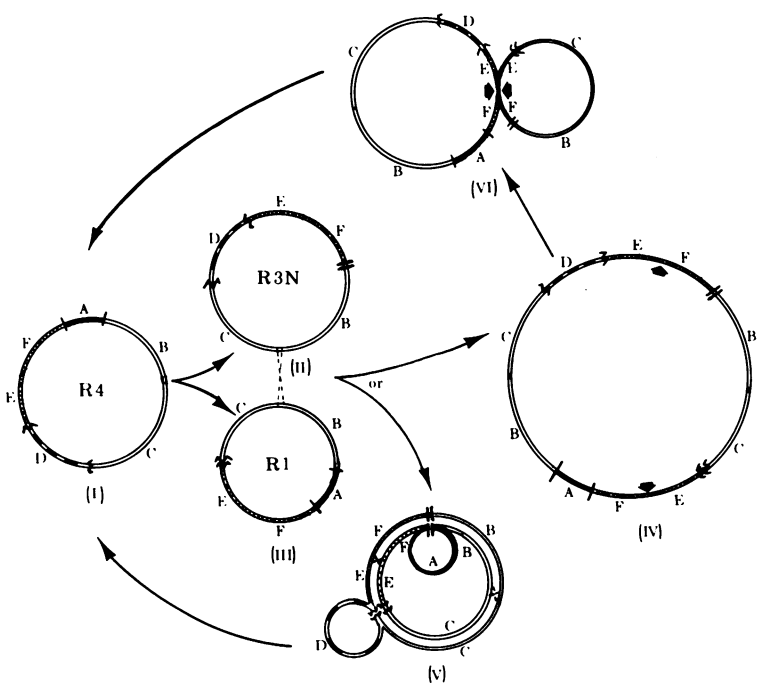

FIG. 13. *Schematic diagram representing possible alternative pathways of recombination of two segregant R factors to reconstitute the parental type. Structure I is the parental 222/R4 factor giving rise to the 222/R3N segregant (II) having lost region A, and the 222/R1 segregant (III) having lost region D. The parental R4 structure could be reconstituted by either (i) two sequential crossing-over events (the first, between B and C, gives rise to structure IV by "addition" of II and III, followed by a further reciprocal crossing-over event between E and F, eliminating the duplicated region BCEF to release the "left-hand" part of VI, identical with I), or alternatively, (ii) two simultaneous crossing-over events (in similar regions as above could occur after the formation of the double-looped structure, V, to regenerate a structure identical to I) (177).*

TABLE 6. *Densities and molecular sizes of R factor molecules found in strains of Proteus carrying 222/R3W, R1, or R6*[a]

Plasmid	Densities (g/cm³)	Contour length (μm) ± SSD	Molecular wt[b] (Mdal)	Reference
222/R3W[c]	1.708	28.5 ± 0.3	59	176
	1.711	35.8 ± 0.3[d]	74[d]	
	1.717	6.4 ± 0.1	13	
R1	1.709	28 ± 1.0	58	50
	1.711	33 ± 0.8	68	
	1.717-8	5 ± 0.5	10	
R6	1.709	26 to 31	54-64	50
	1.711	31 to 38	64-79	
	1.717-8	4 to 7	8-14	

[a] DNA was isolated by either SLS (176) or Brij 58 + SDS (50). It was then centrifuged in CsCl (176 and 50 [R1]) or $CsSO_4$ + Hg^{2+} (50 [R6]). The three molecular sizes of 222 separated from logarithmic-phase cultures are shown in Fig. 14. R1 and R6 fractions at 1.709 to 1.711 density were isolated from logarithmic-phase cultures and 1.717 to 1.718 fractions from stationary cultures (50).

[b] Calculated on the basis of 2.07 Mdal per μm of DNA (147).

[c] Plasmid 222/R3W is a tetracycline-sensitive segregant of 222/R4 (74,236). From contour length data, it appears to be either a point mutant or a small deletion mutant of 222/R4 (177).

[d] Based on 16 molecules, other experimental data measuring a further 22 molecules of mean contour length 33.3 μm (177) leads to an overall mean of 34.3 μm or 71 Mdal.

sions previously drawn from physiological and genetic experiments (7, 9; *see* Table 1).

PHYSICAL INTERRELATIONSHIPS

The similarity of a number of bacterial plasmids, particularly in their sex factor properties, has led to frequent speculation on their interrelationship, origins, and evolution. Genetic homologies have been inferred from reports of the detection of recombinants between nonisogenic plasmids. In some cases, these reports are based on tenuous evidence of cotransfer or, what may now be suspect, cotransduction data (102); in other cases, notably the complex F·ColV·ColB·*trp*·*cys*, the genetic

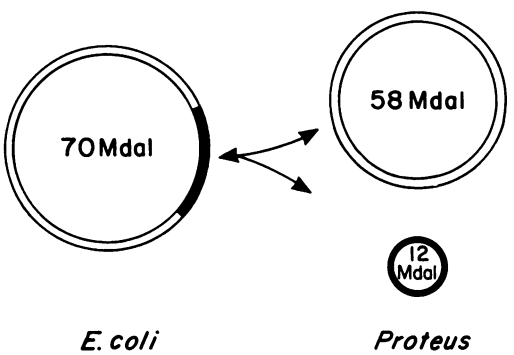

FIG. 15. *Alternate molecular forms of 222/R3W factor as a composite (70 Mdal) molecule in E. coli which separates in Proteus into two component replicons of 58 Mdal and 12 Mdal.*

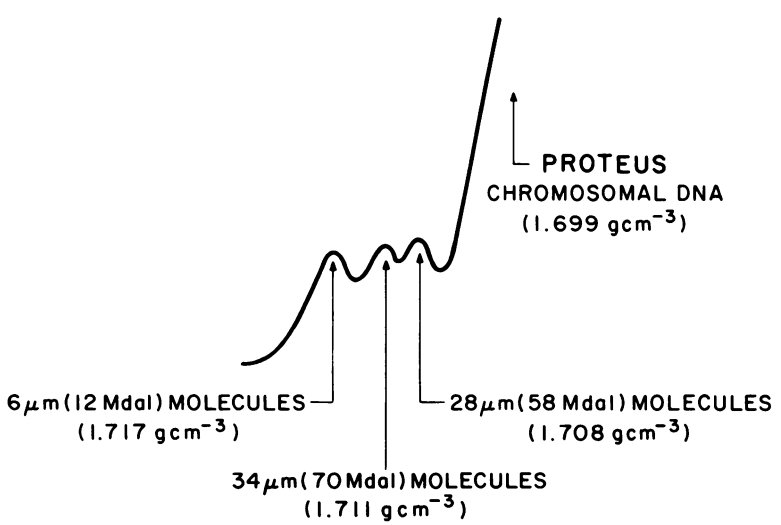

FIG. 14. *Diagrammatic representation of satellite peaks of 222/R3W DNA (enlargement of part of Fig. 7 or 8) isolated from a Proteus host strain after preparative CsCl density-gradient centrifugation, indicating densities and contour lengths (in micrometers) of molecules measured, and calculated molecular weights in megadaltons (from 176).*

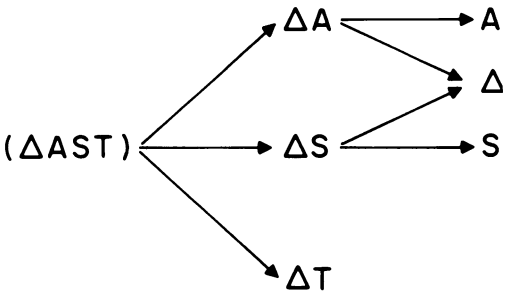

FIG. 16. *Derivation of strains in ΔSAT system. Δ denotes a strain with transfer factor properties; S, A, and T represent noninfectious resistance to streptomycin (plus sulfonamide), ampicillin, and tetracycline, respectively (9).*

TABLE 7. *Contour lengths and molecular weights of a number of plasmids of the ΔSAT system of Anderson and Lewis (9)*[a]

Plasmid	Contour length ± SSD (μm)	No. of molecules measured	Molecular wt (Mdal)
Δ	29.1 ± 0.8	33	60
S	2.9 ± 0.13	63	6.0
ΔS	29.3 ± 1.1	51	61
	2.7 ± 0.13	296	5.6
ΔA	29.5 ± 1.4	32	61
	2.7 ± 0.15	210	5.6
Δ-T	32.3 ± 1.1	80	67

[a] Data from Milliken and Clowes (168a).

evidence (84) has been supported by the physical isolation of a unitary DNA structure (115). Good evidence has also been indicated of physiological similarity of a number of different sex factors through the cross complementation of fertility characteristics (1, 2, 184, 185, 248).

Physical demonstration of genetic homology is possible through DNA:DNA hybridization studies (19). Radioactive DNA of one plasmid is sheared into small fragments of about 300,000 daltons and is then denatured into single strands. The proportion of this label which can bind to sheared, denatured DNA, originating from a different plasmid and immobilized in an agar gel, is measured (73, 75). Early binding experiments led to overestimations of homology, probably because of nonhomologous binding at regions having partial homologies (73). More recent experiments have shown that much of this binding is unstable at temperatures well below the temperature of denaturation of the DNA of either parent, whereas a proportion, presumably representing regions of complete homology, is as stable as a homologous control. Under these more exacting conditions, Falkow and co-workers have shown that R1 has a homology of 74% with 222, 35% with F, and 16% with ColIb (76).

More recently, a technique has been described for examining by electron microscopy the regions of hybridization between two strands of DNA from different origins (64, 218). Two plasmid DNA species of distinctive size are denatured, mixed, and allowed to reanneal. Homologous regions on heterologous duplexes can be seen as double-stranded regions of DNA, whereas the nonhomologous segments remain as single strands which can be distinguished by electron microscopy both by thickness and conformation (*see* Fig. 11b). By using this technique, Cohen and associates (52) have shown that the homologous region of R6 and F comprises 44% of the R factor and is in one contiguous segment. Clearly the use of this technique is destined to play a major role in future studies of relationships between plasmids, particularly if correlations are made between the location of the homologies and the genetic map as has been already so successfully accomplished in the case of λ (78, 218).

MOLECULAR STRUCTURE

Monomolecular Plasmids

It is clear that all plasmids so far examined are circular, double-stranded DNA molecules and exist intracellularly for at least a portion of their time as covalently closed structures. The circularity of plasmids had been anticipated as the structure that would be consistent with their integration as episomes within the host chromosome in a manner analogous to that originally proposed by Campbell for the λ molecule (29), later confirmed to be physically circular (114). However, in view of the fact that integration may not in fact be a general property of plasmids, circularity seems more likely to represent an essential structure for replication (133).

It has been suggested (112) that the covalent ring closure of plasmids may be an artifact produced subsequent to cellular lysis by the action of a bacterial ligase (100), but this appears to be at variance with a number of well-established data (see 89 for discussion), and we will conclude that the CCC form is established intracellularly. The supercoiled structure of plasmids would appear to have no significance of any special plasmid quality but to be merely a consequence of covalent ring closure. Since it seems likely that the number of superhelical twists in all CCC molecules (including plas-

mids) is proportional to the contour length (25, 55), the argument has been made (25) that supertwisting is the extracellular end result of ring closure of intracellular DNA which, perhaps due to the ionic conditions existing within the cell, has a helical pitch slightly less than that measured in the original "B" configuration (159).

In CCC DNA isolated from mitochondria and animal viruses, and double-stranded replicating forms of single-stranded bacterial viruses, a further degree of configurational complexity has been described which results from the interlocking of several CCC molecules ("catenation") forming products termed catenanes, which are usually dimers, but may also be trimers or higher multimers (*see* Fig. 12b). These structures can be identified since one of the interlocked molecules may be "relaxed" or "open," whereas others in the same catenane remain covalently closed. Such molecules would have a density in ethidium bromide-cesium chloride gradients intermediate between CCC and OC molecules and may thereby be isolated and identified (Fig. 17). Catenated DNA was first described in bacterial plasmids isolated from *S. aureus* (205), and later for an enteric bacterial fi^- factor, R6K, controlling penicillinase (145). However, this latter factor produces unusually high amounts of plasmid DNA, and the proportion (5% to 10%) of catenated molecules may thus be more easily detected in this strain, but may be characteristic of all plasmid DNA species. This conclusion has been made more probable by a recent report on the isolation of catenated DNA of R1 and R6 factors (both fi^+) from minicells (48, 52) and of catenated ColE1, also from minicells (93, 130). It has not been possible to correlate the catenation of mitochondrial DNA with an origin by either replication or recombination, and it may well occur subsequent to ring closure by random breakage and rejoining of double-stranded ring structures (124). Recent evidence from $\phi X174$ (17) leads to the conclusion that, although circular multimers arise as a result of replication errors, this is not a source of most catenanes. These are formed by recombination errors which, if nonreciprocal, would be equivalent to the breaking of a circular molecule and its rejoining after interlock with another circular molecule. The amount of catenated DNA found in R6K DNA was remarkably constant, corresponding to one dimer per chromosome, irrespective of the physiological state of the cells, in contrast to the amount of noncatenated CCC DNA which increased threefold in cultures moving into resting phase (145). Catenation of bacterial plasmids may thus be related to a replication or a segregation mechanism involving a membrane structure (133).

A summary spectrum of the molecular sizes of bacterial plasmids is shown in Fig. 18, where it can be seen that they range from approximately 3 Mdal to greater than 100 Mdal. One clear distinction emerges: as might be expected from the identification of a dozen or more genes associated with sex-factor fertility (1, 2, 80, 132, 184, 185, 246, 248, 249), plasmids that are noninfectious are smaller (less than 20 Mdal) than those possessing sex-factor activity, and all plasmids greater in size than 26 Mdal are capable of self-transfer and have fertility characteristics.

The size of the R factors 222, R6, and ΔT relative to the DNA content of the generalized transducing phages P1 (126) and P22 (193) is consistent with the fact that each factor can

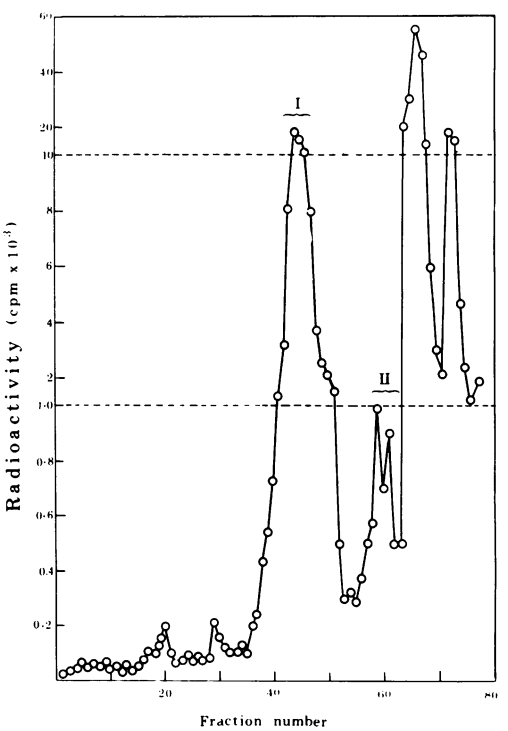

FIG. 17. *Sedimentation profile of DNA from an E. coli (R6K)⁺ culture after ethidium bromide-CsCl density centrifugation. Fractions are numbered as collected in five-drop samples from the bottom of the centrifuge tube. Radioactivity in 0.006-ml samples was determined by liquid scintillation counting of the sample after filtration and washing through a membrane filter. Electron microscopy showed that peak I contained CCC DNA; peak II contained predominantly catenated DNA. (145).*

be transduced in toto by P1 (10, 237, 242) (molecular weight 78 Mdal), but only transduction of parts of each R factor is found with P22 (11, 237, 242) (molecular weight 28 Mdal).

The molecular relationships found for various plasmids do not so far lead to any obvious interpretation for the mechanism of curing. Clearly, all plasmids are supercoiled structures, and since many plasmids can be cured by drugs that intercalate, it has been proposed (104) that curing may depend upon the CCC form. However, this does not seem to take into account differences found in susceptibility to curing agents; for example, the two colicinogenic plasmids ColIb and ColE1 are completely refractory to curing by the intercalating dye, AO, in contrast to the sensitivity of F (46), although ColIb and F are very similar in size and ColE1 is much smaller.

Curing seems to result from an inhibition of plasmid replication rather than any effect on segregation (57, 120). Furthermore, since the *initiation* of chromosomal replication becomes more susceptible to AO inhibition when it comes under the control of an integrated F factor (175), it may be concluded that the greater sensitivity to AO of F replication compared to chromosomal replication (thus leading to curing) may lie in the initiation event. Moreover, rifampin, which appears to prevent the initiation of RNA transcription by binding to RNA polymerase (244), has also been reported to lead to curing in *S. aureus* (137) and more recently to ColE1 in *E. coli* (35). Since the act of transcription rather than the product(s) has been suggested to be a necessary prerequisite for the initiation of DNA replication (68), it may be concluded that "curing" originates in this event and that any compound that preferentially interferes with the initiation of plasmid DNA replication might be an effective curing agent. A similar interpretation would arise if in fact an RNA primer were necessary to initiate the replication of DNA (24*a*).

A Revised Map for R222

The establishment of a monomolecular DNA species for R factor 222 in *E. coli* (177) confirms its genetic structure as a plasmid cointegrate (*see* Fig. 1, Table 1). Values for the molecular weights of the segregant molecules of 222 (177 and Table 5) permit the construction of a revised physical map (Fig. 19) with the chloramphenicol (Cm), streptomycin (Sm)-sulfonamide (Su) elements located within segment CD and the tetracycline (Tc) resistance gene within DE (a similar map can be drawn for R1 and R6 with the *amp*, *kan*, and *neo* markers located in the CDE region). Since both the segregant factors R1A (AXFED) and R3N (FXABCD) appear to have normal transfer factor properties, all genes controlling transfer of these plasmids, sometimes referred to as the RTF region, must be located within the AXF segment. If 222 resembles R1 and R6 so that its 12 Mdal segregant in *Proteus* (CDE segment) can be identified as the segregant carrying the drug-resistance determinants ("r" segregant) and the 58 Mdal segregant (CBAXFE segment) as the drug-sensitive segregant carrying transfer factor properties (RTF), then the latter clearly has more DNA than is required for RTF function, if only because it is larger than R1A which retains RTF properties and also has a Tc resistance region. If RTF occupies a large part of the AXF segment, because of the size of P22 DNA (ca. 28 Mdal), it would not be possible for P22 transductants selected for either Cm or Tc to include region AXF, and they would therefore be noninfectious (237). What is difficult to explain is that, although the Sm-Su, Cm, and Tc determinants (segment CDE) may be concluded to segregate as the 12 Mdal molecule, they are not picked up by P22 to give stable noninfectious Cm^r, Su-Sm^r, Tc^r transductants. (Nor are those transductants that *are* found—$Cm^rSm^rSu^r$ or Tc^r—apparently able to persist even as noninfectious replicating plasmids [70, 237].) This could be due to the inability of the 12 Mdal molecule (or parts of it) to be stably replicated in the absence of the 58 Mdal molecule. Either the 12 Mdal molecule has a replicator locus but no regulation locus, the larger 58 Mdal plasmid molecule providing either some form of positive control in the shape of an initiator, or, as previously suggested (146, 191), a negative (repressor) control which prevents unregulated synthesis of the 12 Mdal element which would otherwise lead to the death of a transductant carrying this element. Alternatively, there may always be a break between Cm, Sm-Su and Tc in the linear molecules that are perhaps necessary for incorporation in P22 for transduction. If these molecules were assumed to arise during replication, it might be concluded that the replicator locus of the composite 70 Mdal 222 molecule was situated between Tc and Cm,Su-Sm.

Alternative Molecular Structures of R Factors

Figure 20 summarizes diagrammatically the molecular nature of R factors which can be

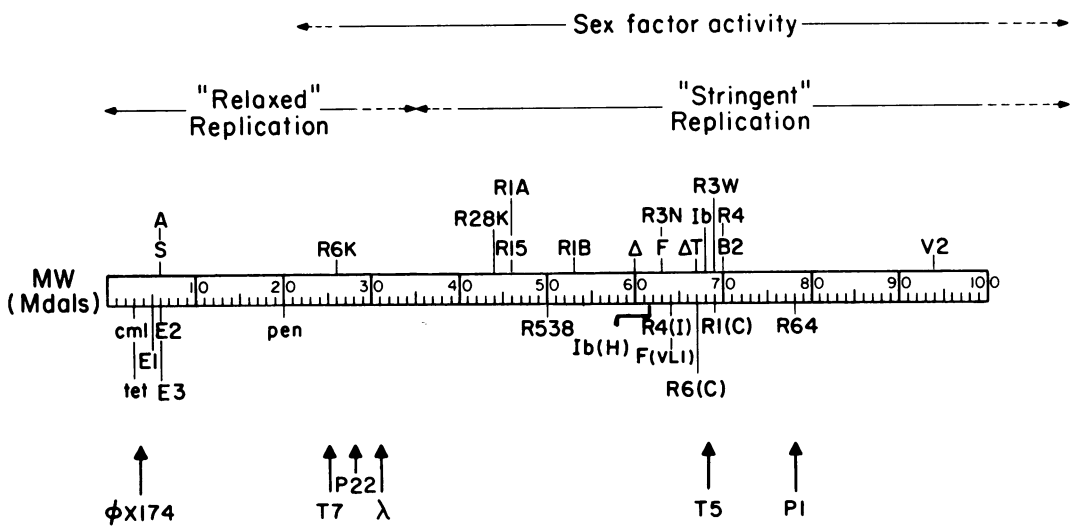

FIG. 18. *Size spectrum of bacterial plasmids. Data of plasmids shown above the scale are those of the author and colleagues, and below the scale those of other workers (detail in Tables 2, 3, and 4; note that R1A, R1B, R3N, R3W, and R4 are abbreviations for derivatives of the 222 R factor; Ib, B2, and V2 are abbreviations for the colicin factors ColIb-P9, ColB2-K77, and ColV2-K94, respectively); pen, tet, and cml are plasmids of S. aureus (179). Ib(H) is size of ColIb from Clewell and Helinski (38), F(VLI) is value of F in reference 227, R4(I) is value of 222/R4 by Inselberg (128). R1(C) and R6(C) are data of Cohen and Miller (50) for the factors R1 and R6. The sizes of a number of phage DNA species are shown by arrows below: φχ174 (79), T7 and T5 (87), P22 (193), λ (61), and P1 (126).*

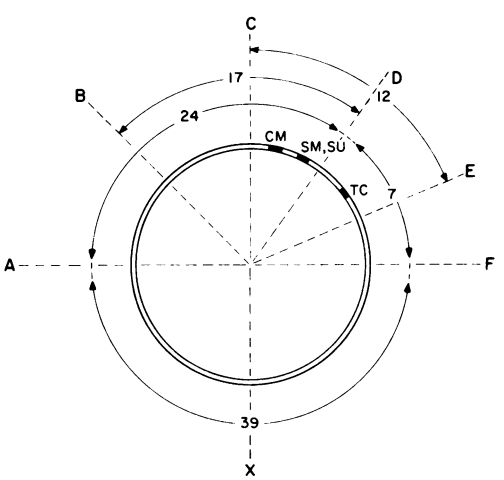

FIG. 19. *Revised physical map of 222/R4 based on contour lengths of DNA from deletion mutants (177) showing sizes in megadaltons. Cm, Sm, Su, and Tc represent the genes controlling resistance to chloramphenicol, streptomycin, sulfonamide, and tetracycline, respectively. From the parental molecule 222/R4 (70 Mdal), deletion AD (24 Mdal) gives R1A (46 Mdal), deletion BD (17 Mdal) gives R1B (53 Mdal), deletion DF (7 Mdal) gives R3N (63 Mdal), and deletion CE (12 Mdal) would give "resistance transfer factor" assuming similar data are found as for R1 and R6 (50, 51).*

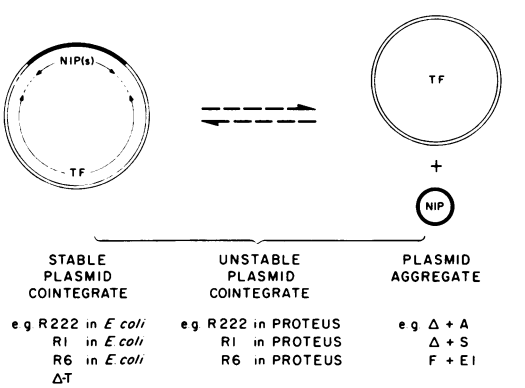

FIG. 20. *Alternative molecular structures of R factors. TF represents genes controlling conjugal transfer, which can exist as an independent structure known as a transfer factor. NIP represents genes controlling other properties which can exist independently as noninfectious plasmids.*

represented by several alternative structures. The major R factor type appears to have a composite structure, in which a number of elements are cointegrated in the same circular plasmid molecule to form a stable "*plasmid cointegrate.*" This structure is clearly appropriate for the R factors 222, R1, R6, and ΔT as they exist in *E. coli*, since both genetic studies

(9, 10, 233, 237, 242) and physical studies (Tables 4 and 7) are consistent with a single structure. It is also consistent for other plasmids such as the F·ColV·ColB·*trp*·*cys* plasmid (from both genetic studies [Fig. 2; reference 84] and physical studies [115]) and for the P1 penicillinase plasmid which has a circular genetic map (Fig. 3; reference 179) and takes the form of a single 19 Mdal circular DNA molecule (179).

In some cases, the cointegrate has incorporated more than one element capable of independent replication and, although stable in one host, it may break down into its independently replicating molecules in another host. Examples of this are the factors 222, R1, and R6, which are stable in *E. coli*, but when transferred to a *Proteus* host strain they act as an unstable plasmid cointegrate and segregate two of their component replicons (*see* Fig. 15) (described by some workers as an "association-dissociation" phenomenon [52, 76, 111]). Since *Proteus* would not appear to be the normal host for these plasmids, it is perhaps not surprising that a measure of control is lost and the plasmid may revert to what may be supposed as a more primitive evolutionary type.

Another type of R factor structure is represented by the "*plasmid aggregate*," as found in ΔA and ΔS, which is similar to the artificial complex F ColE1. In these systems, the transfer factor properties are carried on a large molecule and the drug resistance (or colicinogenic properties) on one (or more) noninfectious small molecule(s) which may frequently be transferred as a result of being resident in the same cell as the infectious plasmid. At the moment, one can only speculate on the physical basis for the conjugal cotransfer of these elements (which has also been referred to as "association" [5, 7]) but, because of the lack of recombination between them in strains which received their plasmids by this transfer (168*a*). The probability of transient covalent linkage would appear to be unlikely. It has previously been concluded that chromosomal segments may be transferred from one bacterial strain to another by virtue of a conjugating system set up by the ColIb sex factor, which appears to be unable to integrate into the chromosome (45, 173), and is therefore not likely to be covalently linked to the chromosomal segments in conjugal transfer. Similarly, transfer of a noninfectious element such as S, A, E1, or E2 by mobilization with either Δ, ColIb, or F (9, 43, 185) may occur in a parallel way and be independent of any physical association between the two elements, both being presumed to be transferred as single-stranded DNA molecules with a 5′-3′ orientation (183, 204).

Speculations on R-Factor Evolution

The idea that R factors are comprised of independent replicons, and therefore that they may arise by de novo mutation of molecules which are initially extrachromosomal, leads to some interesting speculations on their evolution. It seems clear at least that, as an alternative evolutionary origin of R factors by a "gene pick-up" mechanism, we must consider that some drug-resistance genes originate (after mutation?) from elements which were extrachromosomal at the time that selective antibiotic pressure evolved the R factors in the variety and profusion that we see today. Whether drug-resistance genes originate chromosomally or extrachromosomally, the same problem exists of establishing what is the "normal" function of genes that mutate to give resistance to antibiotics that the bacteria is not likely to have had contact with in the course of normal evolutionary development. Since many R-factor resistances are due to such standard enzymatic processes as acetylation, phosphorylation, adenylation, etc. (62, 63, 212) (as opposed to chromosomal antibiotic resistance which results from alterations in ribosomal structure), it is possible to conceive of R factor evolution via the evolution of enzymes through an increasing affinity for an antibiotic as substrate, the original enzyme activity being directed towards a different substrate that is more commonly encountered. Indeed, increasing substrate affinity by evolution has already been proposed by Shaw (212) on the basis of differences in Michaelis (K_m) constants of a number of Cm-transacetylating enzymes from Cm-resistant bacteria.

Since in enteric bacteria most extrachromosomal elements can be mobilized by one or another transfer factors and, indeed, transfer factors of either F-like or I-like type can mobilize the same non-infectious plasmid, the simultaneous presence of transfer factors in the same interfertile population as smaller noninfectious drug-resistance elements would be likely to ensure the rapid dissemination of these smaller resistance molecules, leading to systems such as Δ^+S^+ and Δ^+A^+. This would suggest that the plasmid aggregates may be early evolutionary forms. Later perhaps, some of these plasmid aggregates may have developed into plasmid cointegrates which are

likely to be a more efficient system for both the transfer and the stability of drug resistance.

ASPECTS OF PLASMID REPLICATION

Plasmid Copy Number—Relaxed and Stringent Replication

Since we know the molecular weights of a number of plasmids, and the molecular weight of the host chromosome is also known (the most commonly used value for *E. coli* being 2500 Mdal [53]), an assay of the ratio of plasmid to chromosomal DNA would permit a calculation of the relative numbers of copies of plasmid to chromosome in those cases where only one size of plasmid molecule exists in a cell. A number of methods depend upon the measurement of plasmid molecules as representing the only CCC DNA in the cell on the assumption that most plasmid DNA exists intracellularly in this state. Freifelder et al. concluded that 25 to 100% F'*lac* DNA exists as CCC molecules (89). Clewell and Helinski (39) isolated an unstable plasmid DNA molecule which they argue is lost in the standard procedures for isolating CCC molecules and conclude that CCC DNA accounts for between 27 and 44% of the total plasmid DNA. Hence, the measurement of CCC DNA to chromosomal DNA gives only a rough estimation of the number of plasmid copies, and this will clearly be a lower limit. The percentage of CCC plasmid to chromosomal DNA has been estimated in various ways. Some of the results are shown in Table 8. In spite of the fact that many of these methods are subject to certain apparently uncontrollable experimental variations (89; probably reflecting the proportion of plasmid DNA isolated in CCC fractions), one important feature emerges from this type of data. Table 8 shows that for many plasmids the number of copies correspond to between one and two per chromosome. For the remaining plasmids, this relationship clearly does not hold, and the relative number of plasmid copies per chromosome is much greater and is generally between 10 and 40. These plasmids include the noninfectious elements ColE1, A, and S and, in addition, the smallest factor so far found with sex factor activity, R6K. Thus, although the data are crude, two distinct classes of plasmids can be recognized. First, those preserving a near unitary relationship in copy number between the factor and the chromosome, which have been termed "stringent" in the regulation of their DNA replication (145), and secondly, those where there are 10-fold or so more copies of the R factor than the chromosome, in which the replication of the DNA has been termed "relaxed" (198). A further feature of at least some relaxed-regulated plasmids is that the relative numbers of copies appears to increase some threefold if lysates are made from stationary rather than from logarithmic cultures (139, 145, 198). Moreover, the control of plasmid replication appears to be size-related (Fig. 18), only the smaller plasmids being relaxed (although the discrepancy should be noted between the stringent *pen* plasmid [~20 Mdal] and the relaxed R6K [26 Mdal], which may possibly be due to differences in the staphylococcal and enterobacterial systems). Relaxation thus appears to result from a lack of controlling genes and implies that stringent control may be effected via repressor genes. Clearly, a stringent relationship would appear

TABLE 8. *Amounts of plasmid and chromosomal DNA and ratio of number of copies of plasmid per chromosome*[a]

Replication control	Plasmid[b]	Method[c]	Ratio S/C[d]	Copy no.[e]	Reference
Relaxed	ColE1	Brij	1.3 to 2.3	7 to 12	39
	S	EB	3.1	14	168*a*
			15[f]	13	145
	R6K	EB	40[g]	38	145
Stringent	R28K	EB	4.1	2.2	145
	R15	EB	2.5	1.4	177
	R538	AS	3.3	1.7	227
	λ	EB	1.8	0.8	168*a*
	F	EB	2.2	0.9[h]	15
		AS	1.7	0.7[h]	227
	222/R4[i]	EB	5.0	1.8	177
	R64	AS	3.7	1.2	227
	FVB*tc*	BR	5.0	1.1	115

[a] All plasmids isolated from *E. coli* K-12, except FVB*tc* (Fredericq's F·ColV·ColB·*trp*·*cys*) from *Proteus*.

[b] Arranged in order of increasing molecular weight.

[c] EB, Ethidium bromide-cesium chloride of crude lysate; AS, lysis with Sarkosyl, alkaline sucrose centrifugation (227); Brij, Brij/DOC lysis (36); BR, Sarkosyl lysis of *Proteus* host strain (115).

[d] Ratio of DNA as percent thymine label in satellite peak to chromosomal peak.

[e] Calculated from *d* using molecular weights in Tables 2, 3, and 4 and molecular weight for DNA of *E. coli* 2,500 Mdal, *Proteus*, 2,300 Mdal.

[f] From logarithmic-phase cultures.

[g] From stationary-phase cultures.

[h] Assuming molecular weight of F of 64 Mdal.

[i] DNA from the 222 segregants R3W, R3N, R1A, R1B give rise to a plasmid chromosome copy ratio of 2.2, 1.2, 3.3, and 1.5, respectively (177).

to be more stable and could be a stage in development from relaxed replication towards stabilization by chromosomal integration.

Table 8 shows data only or enteric bacterial plasmids. More limited data for staphylococcal plasmids, obtained by Novick and co-workers (179) by the use of "cleared lysates" and therefore possibly more reliable than that depending on the isolation of CCC DNA, show a striking parallel. The penicillinase plasmid of about 20 Mdal is estimated as being present in two to three copies per cell, whereas the smaller tetracycline plasmid (about 3 Mdal) exists in 30 to 32 copies per cell, and preliminary studies suggest that the small chloramphenicol plasmid (also about 3 Mdal) may also exist in "many copies per cell" (179).

From a limited number of experiments, it also appears that each plasmid copy is active, to give a constant "gene-dosage" response. This appears to be true for the F′ element carrying β-galactosidase activity (134), for the penicillinase gene of R6K (145), and for the Cm-transacetylating activity of R222 (199).

Mechanism of Relaxed Replication

A strain of *Proteus* containing the 222 R factor grown for several generations in the presence of Cm has an R-factor DNA level about 16% that of chromosome. Rownd assumed the R-factor molecular weight to be 50 Mdal which would be equivalent to about 10 plasmid copies per chromosome, and posed the question of how these 10 copies replicated (198). (If in fact the preponderant R factor molecule is the 12 Mdal molecule [*see* Fig. 15], this would lead to a copy number of about 32 but would not influence the following arguments.) Two possible models were proposed. One, a "master copy" model, suggested only one of these 10 molecules replicated, and did so 10 times for each replication of the chromosome, to produce the 20 copies required at cell division. The alternative model, which would be called the "democratic" model, suggests that each of the 10 copies replicated once for each chromosomal replication. Rownd performed a Meselson-Stahl density-shift experiment moving the host strain from a ^{15}N (heavy) medium to a ^{14}N (light) medium and analyzing R-factor DNA at various generation times after density shift. The master copy model would predict that, one generation after shift, nine of the molecules would remain heavy (HH), two would be hybrid (HL), and nine would be light (LL). In contrast, the democratic model would produce all (20) hybrid (HL) R-factor molecules. Rownd analyzed the distribution of DNA in the R-factor satellite band by means of a curve resolver and showed that, after one generation, approximately one-fourth of the DNA was HH, one-half was HL, and one-fourth was LL, satisfying neither of the suggested models and being consistent with the idea of a random replicating pool; after density shift, one molecule from the pool would begin to replicate; during its replication, other molecules in the pool would not replicate; after replication, the two daughter plasmid molecules would return to the pool where the probability of their further replication would be equal to the probability of replication of any one of the other unreplicated molecules (198). It should be noted that these results would not lead to this interpretation if account is taken that the system studied comprises three distinct molecular species (176) and if it is not assumed that the bulk of the DNA is represented by only one of these molecular species. For example, the data are also consistent with 50% of the DNA of either a stringently controlled molecular species that replicates once, or a relaxed controlled species that replicates "democratically," with the remaining 50% of the DNA replicating in a relaxed way from a master copy. However, a similar experiment by Bazaral and Helinski (15) observing the replication of the monomolecular ColE1 species in *E. coli*, with approximately 10 identical plasmid molecules per chromosome, led to an essentially similar conclusion as that of Rownd, favoring a randomly replicating pool.

If we accept the idea of a random replicating pool, these experiments rule out a simple idea of membrane attachment and replication which in its simplest form would have led to a master copy model. Membrane attachment is of course not excluded, but if all molecules were membrane bound, an initiator molecule would be required in limiting amounts so as to permit only one of the attached molecules to replicate at any one time. Alternatively, intermittent attachment and replication of a random molecule from the pool, the subsequent detachment of the two daughter molecules and their return to the pool before the next signal for attachment, though cumbersome, would also preserve the idea of membrane attachment. Kasamatsu and Rownd (139) also concluded from the observations that growth under one of several physiological conditions rapidly led to a characteristic

number of replications per generation and that replication was under a simple, positive control.

"Transition" of Drug Resistance

In strains of *Proteus* carrying R factor 222, Rownd also reported a phenomenon which he termed "transition" (199, 202). When such 222^+ strains are grown for a number of generations in either Cm-, Sm-, or Su-containing medium, to which the R factor confers resistance (but not in Tc-containing medium to which it also confers resistance), the density of the satellite band shifts, so that after 200 generations it is almost entirely of density 1.718 g/cm^3 and of an amount corresponding to approximately 30% that of the chromosome. When the culture medium is shifted to exclude any antibiotic, sequential samples show a decrease in the 1.718 g/cm^3 satellite and the appearance of a 1.711 g/cm^3 satellite. After approximately 200 generations in drug-free medium, there is little if any satellite DNA of density 1.718 g/cm^3, and the total satellite DNA is restricted to density of 1.711 g/cm^3 and is reduced to about 6% that of the chromosome (199, 200). Other workers have obtained similar data for growth in drug-free and drug-containing medium (146, 191). As previously shown, in *Proteus* $(222)^+$ cultures grown directly from a single colony, three sizes of molecules of 12 Mdal (1.717 g/cm^3), 58 Mdal (1.708 g/cm^3), and 70 Mdal (1.711 g/cm^3) are present (*see* Fig. 15; 176), and similar conclusions have been proposed from studies with two independent R factors R1 and R6 (50, 75). Since the 1.717 g/cm^3 peak (Fig. 14) is equal to or greater in size than the other density peaks and is comprised of smaller molecules than those in the other peaks, the 12 Mdal molecules must be present in more copies than the 58 Mdal and the 70 Mdal molecules (176). These results have been confirmed by other workers (146) and have also been found for R1 or R6 (50, 75). Other small plasmid molecules are relaxed in their DNA replication, in contrast to larger molecules which are stringent (Table 8 and Fig. 18). These data are thus consistent with the idea that the 12 Mdal molecule is relaxed and the 58 Mdal and 70 Mdal molecules are stringent. Moreover, if the 58 Mdal molecule is identified in 222, as it has been in R1 or R6, as a drug-sensitive segregant carrying RTF (51, 216), then the 12 Mdal molecule would carry all four drug resistances. With the R6K plasmid, the level of drug resistance increases in parallel with R factor copy number (145). An increase in the copy number of the 12 Mdal plasmid would thus be expected to lead to increased resistance to those drugs where resistance is mediated through an antibiotic-inactivating mechanism (e.g., Cm, Sm, and Su) but not to an increase in resistance which may be due to a mechanism depending on a cell wall permeability change (Tc^r; see references 62 and 212). "Transition" would then be consistent with an increase in the relative numbers of copies of the 12 Mdal molecule in drug-containing medium leading to a preponderance of DNA at the density of this molecule (1.717 g/cm^3; variations in experimental reproducibility of density peak measurements are about ± 0.001 g/cm^3). "Back-transition" in drug-free medium would be due to a reduction in the number of copies of the 12 Mdal molecules until they were equal in number to the 58 Mdal molecule (1.708 g/cm^3) or perhaps even integrated as part of the 70 Mdal (1.711 g/cm^3, composite) molecule, when they would be subject to the stringent control imposed by the other (58 Mdal) component of this molecule. The interpretations of this phenomenon by Rownd and coworkers (63, 199, 200, 202) appear to ignore certain aspects of the molecular studies with 222 (176, 177) and of the related factor R1 (49, 50, 51, 103, 215, 216) and R6 (50, 52) and are not consistent with what is known of the regulatory control of other plasmids (Table 8 and Fig. 18). Rownd's interpretation similarly identifies the RTF component with a large molecule of 1.711 g/cm^3 and the drug resistance (r) elements as small molecules of density 1.718 g/cm^3. However, r is concluded to be stringently controlled in the cell, whereas RTF is assumed to be relaxed and present in multiple copies; the explanation of the density change in "transition" being based upon the elaboration of large polymeric molecules comprised of a single copy of the RTF (1.711 g/cm^3:70 Mdal) determinant linked to a repeating sequence of a large number of r (1.718 g/cm^3:12 Mdal) determinants. These molecules would need to approach molecular weights of several hundred megadaltons to account for the density shift and plasmid DNA fractions found, and they have not so far been identified in structural studies, at least not as a common circular DNA molecule. Rownd also concludes that control of R-factor replication is positive and is made through initiator molecules (198, 199).

Kopecko and Punch (146, 191) have claimed

that a similar increase in the ratio of the 1.718 g/cm³ to 1.710 g/cm³ DNA also occurs under various conditions of protein inhibition. They also interpret their data on the basis of a 1.710 g/cm³ (RTF) molecule and a 1.718 g/cm³ (r) molecule and conclude that both are subject to negative control by a repressor produced by a gene on the RTF molecule. In addition, the RTF molecule is proposed to be membrane-attached and subject to positive control. Both molecules are concluded to be present as multiple copies, the r molecule always outnumbering the RTF molecule. Their model accommodates the molecular structural data of 222 and is not inconsistent with what is known of the regulatory control of other plasmids. The lack of a segregant harboring only the 1.718 g/cm³ element (or perhaps more forcibly, the lack of any transductant harboring Cm, Sm, Su, and Tc resistance [237] which would be expected to arise were the 1.718 element able to exist independently as an r element) was interpreted as due to lack of a repressor produced by RTF and necessary to prevent uncontrolled replication of r leading to its lethality for the cell.

(This, however, could be equally well explained by assuming the production of an initiator by the RTF molecule which acts on RTF as well as on the r molecule and can give rise to a number of initiating events. This initiator would normally be controlled by a cellular repressor so that, in *E. coli*, for example, the repressor would inactivate initiator in excess of that required to permit one R-factor replication per cell generation and would thus effectively limit the R factor to the composite molecule. In *Proteus*, this cellular repression would be less effective, so that more initiation events could occur per cell division. Thus, if initiation of molecules was random, segregation into the RTF and r molecules would be likely to lead to a preferential increase in the number of copies of the r molecules, since its time of replication is only about one-fifth that of the larger molecule. Under conditions of drug-resistance selection [transition], those cells where a greater proportion of R-factor DNA was in the form of the r molecules would, because of higher resistance, tend to be selected. Under conditions of protein inhibition, repression would be further reduced and more copies of both molecules would result.)

The replication behavior of 222 discussed above is paralelled by that of ColE1. Recent evidence (34) has been presented to show that, in the presence of Cm, ColE1 continues to replicate for 10 to 15 hr, long after chromosomal replication has ceased, approaching a maximum rate of eight times normal and leading to about 3,000 copies per cell.

DNA-Protein "Relaxation Complexes"

Further work by Helinski and colleagues on the CCC molecules isolated in cleared lysates using Brij and DOC indicates that the molecular structure of such CCC molecules differs from those isolated by ethidium bromide (36, 39). The Brij-isolated CCC molecules from $(ColE1)^+$ cells sedimented in neutral sucrose at 24*S* (the OC derivative sedimenting at 18*S*) compared with values of 23*S* (and 17*S*) for similar molecules isolated after Sarkosyl lysis and ethidium bromide-cesium chloride centrifugation (197). When the 24*S* CCC molecules isolated from cleared lysates were exposed to one of a number of compounds including ethidium bromide, proteolytic enzymes, ionic detergents, or alkali, a large fraction of the CCC DNA was converted to OC DNA, whereas similar treatments were without effect on the 23*S* Sarkosyl-isolated CCC molecules. Further analysis led to the conclusion that, in the intracellular state, a protein was attached to one of the DNA strands of many of the CCC molecules, this CCC DNA-protein complex being termed a "relaxation complex," since removal (or activation) of the protein by one of the above agents led to the breakage of the DNA strand attached followed by "relaxation" of the CCC form into the OC form. It was suggested that the relaxation complex might play a role in the normal replication of the plasmid and that the protein could be an inactive endonuclease (initiator?), activated by the various treatments to produce a nick in one of the strands of DNA (36). Separation of the relaxed (17*S*) complex by alkaline sucrose sedimentation into circular (C) and linear (L) single strands shows that the molecular weights and amounts of both are similar and indicates that only one nick occurs in one of the strands of each molecule. DNA:DNA hybridization of C and L strands from two independent preparations indicated that a specific strand was nicked. More recent work has investigated the sedimentation of the C and L strands in the presence of poly(U,G) (39). Poly(U,G) is known to bind preferentially to DNA strands rich in AT bases (12), and when λ DNA is denatured and centrifuged in poly(U,G), it can be separated into heavy and light strands which are complementary to each other (123). A similar

resolution of a heavy and a light strand was found with DNA from ColE1 (39). Relaxation complexes have also been isolated from ColE2 and ColE3 (18, 37), ColIb (38), and F (144), and in all cases so far studied (ColE1, ColE2, and F) the L strand was heavy and the C strand was light. Thus the heavy strand is in each case the unique strand that is nicked as a result of relaxation.

Replication in Minicells

If a DNA precursor with a radioactive label is incubated with a preparation of minicells that have been separated from a parental strain carrying a colicin factor or R factor, incorporation of label occurs into the DNA of the minicell, previously shown to be entirely plasmid DNA (127, 128, 129, 156-158). The extent of incorporation varies with the plasmid under observation. If a density (5-bromodeoxyuridine, "heavy") label was used, then at 3 hr after the addition of label to minicells containing the 222 R factor, only a small fraction of their DNA was fully dense (consistent with two sequential plasmid replications) and the bulk of the plasmid DNA was found at a hybrid density indicating that most of the 222 R-factor molecules replicate only once in minicells (128). In contrast, minicells containing ColE1 appear to replicate DNA more extensively, so that a greater proportion of the DNA is fully labeled although the majority of the plasmid DNA still remains at the hybrid level (127-129). Thus, growth in minicells reflects the relaxed and stringent replication of the ColE1 and 222 R plasmids, respectively, in the parental (maxi) cells (*see* Table 8).

In the presence of AO (50 μg/ml), which can efficiently cure maxicells of the F factor but which does not cure ColE1 (46) and has little curing effect on the R factor (236), replication of both ColE1 and R DNA species was inhibited to the same relative extent, both being reduced to about 25% of the level of replication found in minicells in the absence of AO (128). (It should be noted that the cell division of maxicells and thus presumably chromosomal replication is also inhibited at this level of AO [116]. The effect of AO on DNA replication in minicells of the F factor or some other plasmid more susceptible to AO curing would appear to be a more crucial experiment.)

Density labeling of minicells has also been used to isolate ColE1 molecules in the process of replication, fractions being selected from each side of the hybrid peak, i.e., that containing only hybrid ("half-heavy") molecules. On examination by electron microscopy, approximately 3% of the circular molecules from these fractions were θ-like in appearance. In all cases, two arms of the (replication) loop were of equal length, and the sum of the length of one of these arms together with the length of the rest of the molecule was a constant, equivalent in contour length to that of the circular ColE1 molecule previously measured, i.e., 2.3 μm. The size of the (replication) loop varied between approximately 3 and 95% that of the total molecule (129). The data are therefore consistent with the idea that the θ structures are in fact replicating molecules consistent with a Cairns (28) symmetrical model. However, a minority of more complex structures were also found as circular 2.3-μm molecules with linear tails of an equal or multiple length (93, 129, 130), consistent with the intermediates of "rolling circle" replication (95).

Transfer Replication

A further aspect of the replicon model (133) proposed that the formation of cellular contacts during conjugation triggers replication and consequent transfer of the donor F sex factor due to its attachment to the membrane. In Hfr cells, the ensuing transfer would thus be due to replication initiated on the sex factor and continued along the continuity of the Hfr chromosome. This idea has recently been vindicated in elegant experiments by Vapnek and Rupp with F^+ donors (228). Using a modification of the Freifelder (90) technique, they were able to isolate CCC DNA selectively from either donor or recipient cells before or after mating. The CCC DNA was then heated for a short time in alkali so as to produce a small number of single-strand breaks in each of the strands and to denature the DNA duplex into its component single strands. The limited number of breaks resulted in the circular strands being broken once only to form intact linear strands. The linear strands were then centrifuged in CsCl in the presence of poly(U,G), under which conditions F-DNA also was found to separate into heavy and light strands. Subsequent analysis of DNA from donor and recipient cells was consistent with the idea that on conjugation a single break occurred in the heavy strand of the CCC F-DNA molecule in the F^+ donor; this heavy linear strand was then transferred to the recipient (by analogy with Hfr transfer, presumably with a 5'-3' orientation [183, 294]), where the light comple-

ment to it was synthesized, at the same time that the heavy complement to the light circular strand remaining in the donor was also synthesized (228). Later experiments have shown that in the case of two R factors, R538 (F-like) and R64 (I-like), similar events occur, in both cases infectious plasmid transfer resulting in the heavy strand being transferred to the recipient (227).

The transfer of the heavy strand in each of these three cases could be coincidence. However, breakage of the heavy strand was found on relaxation of the DNA-protein complex of each of three factors examined by Helinski and colleagues. These data, taken together, would suggest that relaxation of the complexes as seen by Helinski may be the first step in replication which in the case of sex factors also occurs prior to their transfer as well as in normal vegetative replication.

It would be of interest to determine whether transfer of the heavy strand also occurs from those plasmids, such as ColE1 or A, which are noninfectious and are transferred only when coresident with a transfer factor such as F, ColIb, or Δ. If this were so, then cotransfer could be thought to arise by the act of cell contact triggering breakage of the heavy strand of both plasmids, transfer of any single-stranded DNA being semiautomatic under the conditions favoring transfer effected by the sex factor. The quantitative differences of cotransfer observed (e.g., ColE1 by F > ColE2 by ColIb > ColE1 by ColIb > ColE2 by F) in previous experiments (43) might perhaps depend on the relative proximity of the sites of cellular attachment of the plasmids so as to lead to the probability of breakage by the same contact event, or perhaps on the lack of absolute specificity of an endonuclease, which is released by cell contact, acting on the sex factor to initiate transfer replication. A proposal to explain chromosomal transfer by nonintegrating sex factors such as ColIb (45) could also be accommodated within such a model.

Some recent experiments by Falkow and coworkers (76) throw more light on the kinetics of transfer replication and its relation to membrane attachment. Using a fertility-derepressed R1 factor and a conjugation system comprising a non-thymine incorporating donor with an ultraviolet-irradiated, non-DNA-synthesizing recipient (90), specific labeling of a transferred plasmid could be accomplished, and the plasmid DNA could be isolated after either SDS or Brij treatment. Immediately after conjugation, the sex factor in the recipient was found to be membrane-bound, with little evidence of circularity. Shortly thereafter, the label was released from the membrane and began to appear in a sedimentation fraction characteristic of an OC structure and later in a fraction characteristic of CCC molecules. The data are consistent with the transfer of a linear molecule which attaches to the membrane of the recipient, where its complementary strand is synthesized. The double-stranded structure is then circularized and released from the membrane into the cytoplasm as an OC structure, after which covalent ring closure occurs. These experiments were extended to show that entry exclusion by an isogenic or closely related plasmid in the recipient is due to an inhibition early in the replication process so as to prevent membrane attachment, and, even under circumstances where entry exclusion was circumvented by use of aeration to produce R^- "phenocopies," incompatibility was manifest as a reduction in total DNA replication of the transferred plasmid and by the appearance of uncharacteristic sedimentation fractions, not representative of the OC and CCC structures usually found in F^-R^- recipient cells.

CONCLUSIONS

The study of bacterial plasmids can be justified at two levels. In the control of infectious diseases, it is obviously necessary to understand the basis of the rapid proliferation of such elements as infectious drug-resistance factors with a view to curtailing their further spread and attempting to reduce both their incidence and their limitations of antibiotic chemotherapy. More importantly perhaps, plasmids may be a fundamental entity of many bacterial species, involved in "protective" activities other than infectious drug resistance, and they may well have their counterparts in cells of higher organisms.

Central to the further study of bacterial plasmids is an understanding of their replication and segregation. The molecular nature of bacterial plasmids is now sufficiently well established so that more meaningful questions of replication can be posed. Such information is critical to an understanding of curing and incompatibility relationships. The further establishment of cross-homologies between plasmids that are likely to come from electron microscopy studies of heteroduplex formation (64),

together with related studies on fine genetic structure, will ultimately play a major role in the unraveling of the evolutionary relationships. The key problem, however, remains: the role of replication and the role played by the cell and the cell membrane. The relationship of stringent and relaxed control to membrane control is at present difficult to assess. For example, apparent differences in membrane or cell attachment could be inferred by differences in the segregation into minicells of different stringently regulated plasmids including F. F acts as might be expected of a plasmid with a fixed cellular site and is not efficiently segregated to minicells (140), whereas other factors apparently segregate so efficiently as to appear in all minicells (127, 128, 156, 157, 195, 196). The control of F replication appears to be different from that of chromosomal replication, its initiation being uncoordinated with chromosomal initiation, so that over a range of generations times, irrespective of the number of chromosomal initiations and their incidence in time during the course of the cell cycle, F initiation is unchanged and occurs in the middle of the cell cycle (253). Furthermore, the replication of newly transferred penicillinase plasmids in *S. aureus* is not immediately accompanied by hereditary stabilization, and the two events are separated in time and thus may be distinct events (180). Observations on "cleared lysates" obtained by the use of Brij-DOC may indicate that all plasmids so far examined, including F, can be freed of attachments to cell components (111), often inferred to be membranes (96), under conditions where membrane attachment of the chromosome is maintained. Conflicting evidence of "membrane-attachment" has been reported using a different method of preparation (the M′ band method [226]—by direct lysis of lysozyme-EDTA spheroplasts with Sarkosyl on sucrose gradients. DNA in minicells derived from an R factor, or from F·ColV·ColB·*trp*·*cys* (neither plasmid investigated in "cleared lysates") or from an F^+ parent was concluded to be membrane associated from its co-sedimentation with the M band (213).

Promising new approaches have come from studies (57, 179, Kingsbury and Helinski, Bacteriol. Proc., 1970, p. 55; 247) of temperature-sensitive mutants of plasmid-carrying strains, i.e., a class unable to continue plasmid replication at an elevated temperature (40 C) but able to replicate normally at a temperature below normal (30 C). Some of these mutations are located in the plasmid and others in the chromosome. The ability of other wild-type plasmids to complement the plasmid mutants, or be affected by the same chromosomal mutation, offers a key to the understanding of the interrelationship of plasmids of various groups and its influence on incompatibility, fertility repression, and other characteristics. Maintenance of the small ColE1 plasmid has also been shown to depend on the Kornberg polymerase (142), and it may also need a diffusible, chromosomally determined (by the *dnaA* locus) initiator for replication (97). More strikingly perhaps, bacterial mutants temperature sensitive in their DNA replication, which have been shown to belong to the fast-stop *dnaE* group and which are now known to produce a temperature-sensitive DNA-polymerase III enzyme (94) (suggested to be *the* bacterical replicase), may continue to replicate ColE1 at the nonpermissive temperature (98). The specific regulation of some (or all) bacterial plasmid replication through the use of specific replicating enzymes (DNA polymerases I, II, or III, and others unknown) is thus an intriguing possibility.

One of the more interesting recent developments in the study of bacterial plasmids has been the establishment of the class of R-factor plasmid aggregates. Although many R factors are unitary DNA molecules (plasmid cointegrates) controlling both drug resistance and transfer properties, others are represented by the simultaneous presence in the same cell of two or more autonomous replicons differing in physical properties and in the genes carried, exemplified by the Δ (resistance transfer) factor and the r (drug-resistance determinant) elements A or S and probably present in other natural systems (220). Moreover, the breakdown of certain cointegrates into component replicons indicates an intermediate stage between these two types and suggests an evolution whereby aggregates may evolve into cointegrates.

The model of R-factor plasmid aggregates evolving through mutation of extrachromosomal elements might have some virtue as a general mechanism for bacterial evolution. Most bacterial genes are normally not active. They are switched off, except in special environments where their functions are needed. The frequency with which different functional activities are required is likely to vary enormously. If a particular activity (determined by a number of sequentially acting enzymes) is required very infrequently, the necessity to replicate the determinant gene(s) at every rep-

lication of the chromosome may in fact impose more of an evolutionary burden on the species than is compensated for by the rare need for the function. However, if the determinant genes were carried on a plasmid, present in only a few cells per population, their replication would be necessary in only those rare cells. Yet, with the imposition of a need for the activity, these genes could be acquired by all the cells in a progeny population, if the plasmid also had sex factor properties (i.e., was a plasmid cointegrate), or, more economically still, if other cells contained transfer factors which could interreact to establish a "plasmid aggregate." The control of a number of activities in this way by their distribution to a number of different plasmids carried by different cells in the population would enormously increase the total gene pool and extend the overall metabolic potential of the species. It would appear to represent a logical extension of genetic regulation, from the present concept of cellular control of protein synthesis to the species control of gene-pool synthesis. The ability of such a control system to function by responding to selective pressure appears to be well demonstrated in the case of infectious antibiotic resistance in pathogenic *Salmonella* (6). Moreover, enzymes which are concerned in catabolic systems rather than protective systems have recently also been shown to be located on plasmids in *Pseudomonas*—a species noted for its nutritional versatility. A CAM plasmid carrying inducible enzymes for the conversion of D-camphor to isobutyrate has been described in *Pseudomonas putida* and is self-transmitted to other pseudomonads, including *P. aeruginosa*, *P. fluorescens*, and *P. oleovorans* (Chakrabarty and Gunsalus, Bacteriol Proc., 1971, p. 46) and more recently, a similar SAL plasmid controlling salicyclic acid breakdown (Chakrabarty, Abstr. Meeting Amer. Soc. Microbiol. 1972, p. 60). These systems may be concerned with rarely required functions or, as Chakrabarty and Gunsalus (32) describe it, "enzymes concerned with peripheral metabolism." The presence of fertility factors has been known for many years in pseudomonads (121, 122), and R factors have recently been noted in this genus (60, 224), which would further implicate it as a possible reservoir of plasmids.

Bacterial plasmids have often been regarded as a virus subclass of rather lesser importance, but their properties are now divergent enough to consider them as a class within their own right. Although they resemble integrating temperate phages by their ability to establish "symbiosis at the genetic level," they may be thought to be more successful in this role. Their genetic symbiosis, being frequently of the nonintegrated kind, may be less intimate, but it is probably less restrictive and is limited perhaps only to a DNA sequence able to exist as a stable replicon. Their dissemination does not depend upon the destruction of their host, and their transfer may be thought to be more subtle yet more direct. The elaboration of a complex system of proteins, often abandoned once a virus achieves its end of transferring its DNA into a new host, is replaced by the synthesis of those relatively few proteins required for the conversion of the host strain into a donor for the plasmid and is further reserved for emergency use so that the host is not diverted into this activity without purpose. More importantly, the benefits brought to the host may well outweigh those of mere defensive survival and may embrace such a range of catabolic activities as to qualify bacterial plasmids as true small "supernumerary chromosomes" (110), and as such a vital part of the gene pool of the species.

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